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Catecholamine biosynthesis and secretion: physiological and pharmacological effects of secretin

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Abstract

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Pituitary adenylyl cyclase activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) augment the biosynthesis of tyrosine hydroxylase (TH). We tested whether secretin belonging to the glucagon/PACAP/VIP superfamily would increase transcription of the *tyrosine hydroxylase (Th)* gene and modulate catecholamine secretion. Secretin activated transcription of the endogenous *Th* gene and its transfected promoter (EC_{50} ~4.6 nM) in pheochromocytoma (PC12) cells. This was abolished by pre-treatment with a secretin receptor (SCTR) antagonist and by inhibition of protein kinase A (PKA), mitogen-activated protein kinase, or CREB (cAMP response element-binding protein). In agreement, secretin increased PKA activity and induced phosphorylation of CREB and binding to *Th* CRE, suggesting secretin signaling to transcription via a PKA-CREB pathway. Secretin stimulated catecholamine secretion (EC_{50} ~3.5 μM) from PC12 cells, but this was inhibited by pre-treatment with VIP-preferring receptor (VPAC1)/PACAP-preferring receptor (PAC1) antagonists. Secretin-evoked secretion occurred without extracellular Ca^{2+} and was abolished by intracellular Ca^{2+} chelation. Secretin augmented phospholipase C (PLC) activity and increased inositol-1,4,5-triphosphate (IP_3) levels in PC12 cells; PLC-β inhibition blocked secretin-induced catecholamine secretion, indicating the participation of intracellular Ca^{2+} from a phospholipase pathway in secretion. Like PACAP, secretin evoked long-lasting catecholamine secretion, even after only a transient exposure. Thus, transcription is triggered by nanomolar concentrations of the peptide through SCTR, with signaling along the cAMP-PKA and extracellular-signal-regulated kinase 1/2 pathways and through CREB. By contrast, secretion is triggered only by micromolar concentrations of peptide through PAC1/VPAC receptors and by utilizing a PLC/intracellular Ca^{2+} pathway.

Keywords

Secretin; Pituitary adenylyl cyclase activating polypeptide; Vasoactive intestinal polypeptide; Tyrosine hydroxylase; Catecholamine; PC12 cells; Cell culture

Introduction

Secretin, a basic 27-amino-acid-residue carboxy-terminally amidated peptide, was the first hormone discovered by Bayliss and Starling in 1902 (Bayliss and Starling 1902). It belongs to the secretin/glucagon/vasoactive intestinal peptide (VIP)/pituitary adenylyl cyclase-activating polypeptide (PACAP) superfamily and acts as a pleiotropic hormone (Chey and Chang 2003; Chu et al. 2006; Ulrich et al. 1998). Secretin regulates exocrine secretion from the pancreas, gall bladder, and stomach (Mutt 1980; Ulrich et al. 1998). In addition to the regulation of gastrointestinal function, secretin acts in the nervous system and in the cardiovascular system. Thus, secretin induces the excitability of neurons of the nucleus tractus solitarii (Yang et al. 2004). Furthermore, the peptide causes the dose-dependent activation of tyrosine hydroxylase (TH) activity in rat superior cervical ganglia (Ip and Zigmond 2000), pineal gland (Schwarzschild and Zigmond 1989), hypothalamus (Babu and Vijayan 1983), and thoracic paravertebral sympathetic ganglia (Schwarzschild and Zigmond 1991).

We have found that a common variation in the proximal promoter of the tyrosine hydroxylase gene (*Th*; encoding the rate-limiting enzyme in catecholamine biosynthesis) contributes to heritable alteration in many autonomic traits, both biochemical and physiological, and the ultimate disease trait of hypertension (Rao et al. 2007). Members of the secretin superfamily (PACAP and VIP) augment the transcription of *Th* in primary cultures of porcine/bovine chromaffin cells (Isobe et al. 1996; Park et al. 1999; Tonshoff et al. 1997), pheochromocytoma (PC12) cells (Corbitt et al. 1998, 2002; Yukimasa et al. 1999), and central TH-producing cells (CATH.a cells; Muller et al. 1997). Wessels-Reiker et al. (1993) have shown that secretin activates *Th* gene expression in PC12 cells, and that such activation requires cAMP and protein kinase A (PKA); they have however not investigated the signaling pathways downstream of PKA. In the present study, we have found that secretin, at a physiological concentration (low nanomolar), activates the transcription of the *Th* gene, but that this is completely abolished by a secretin receptor (SCTR) antagonist.

Because VIP or PACAP trigger catecholamine release from superfused rat adrenal gland (Malhotra et al. 1988; Przywara et al. 1996; Wakade 1988; Wakade et al. 1991) or from PC12 cells (Taupenot et al. 1999; Taupenot et al. 1998), we have reasoned that secretin might stimulate a similar process in PC12 cells. Like PACAP and VIP, secretin induces catecholamine secretion; however, in contrast to transcription, higher pharmacological concentrations of the peptide are required (low micromolar) to mediate this effect. In contrast to transcription, a SCTR antagonist fails to inhibit the secretory stimulation process. However, both VIP-preferring receptor (VPAC1) and PACAP-preferring receptor (PAC1) antagonists inhibit secretin-induced catecholamine secretion, indicating that secretin triggers secretion through VPAC1 and PAC1 receptor activation. In the present study, we have also dissected the signaling pathways that mediate the transcriptional and secretory responses of secretin in PC12 cells.

Materials and methods

Cell culture and transfection

Cell lines—An early passage (passage 10) of rat PC12 cells were used.

Transient transfection and co-transfection—A rat *Th* promoter (4.5 kb)/luciferase reporter construct was obtained from Dona Chikaraishi (Duke University Medical Center, Durham, N.C., USA). pRSV-PKA inhibitor (PKI), the expression plasmid for the heat-stable inhibitor of cAMP-dependent PKA, was kindly supplied by Richard A. Maurer (University of Iowa, Iowa City, Iowa, USA). We also obtained a dominant negative point mutant of human CREB (cAMP response element-binding protein), namely KCREB (R287L), which was subcloned into the RSV promoter-driven pRC expression vector, from Richard H. Goodman (Vollum Institute, Oregon Health Sciences University, Portland, Ore., USA). PC12 cells were transfected with the *Th* promoter/luciferase reporter constructs with and without co-transfection with the PKI plasmid or CREB inhibitor plasmid KCREB by using the polycationic method (Superfect; QIAGEN) as described previously (Mahapatra et al. 2003). At 4–5 h after transfection, PC12 cells were treated with human secretin (0.0001–1 μM) or saline for 18 h. In time-dependence studies, transfected cells were treated with secretin (1 μM) for 1, 3, 6, 18, or 24 h before harvesting. Transfected cells were also treated with chemical inhibitors of PKA (H-89, 10–20 μM) or extracellular-signal-regulated kinase

(ERK: 5-iodotubercidin, 0.1–2 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) or saline for 18 h before harvesting for luciferase assay. The cell lysates were assayed for luciferase and total cellular protein as described previously (Mahapatra et al. 2003).

Th mRNA expression: real-time reverse transcription followed by polymerase chain reaction—RNA was isolated from control and secretin-treated (1 μM for 18 h) PC12 cells by using the RNeasy Mini kit (Qiagen, Valencia, Calif., USA) and quantified, and 0.5 μg samples were used for reverse transcription (RT). cDNA was synthesized by using iScript Reverse Transcriptase (BioRad, Hercules, Calif., USA) and iScript Buffer at 42°C for 30 min. Real-time RT-polymerase chain reaction (RT-PCR) assays of the *Th* mRNA in PC12 cells were carried out with coding-region-specific primers for rat *Th* (forward: 5′-AGCGCCCATTCTCTGTGAAG-3′; reverse: 5′-GGTGTGAGGGCTGTCCAGTAC-3′). SYBR Green Master Mix (Applied Biosystems, Calif., USA) was used in these assays. To test for contaminating genomic DNA in the RNA preparation, the product of a control RT reaction without reverse transcriptase (−RT control) was treated to amplification by real-time PCR. In all cases, the -RT control did not amplify, even after 40 cycles. ΔCt values were calculated by using D-glyceraldehyde-3-phosphate dehydrogenase as the internal normalization control.

Secretion of catecholamine

Norepinephrine secretion was monitored (as ^{3}H-norepinephrine) as described previously (Mahata et al. 1996). Net secretion was calculated as the secretagogue-stimulated release minus basal release, where basal norepinephrine release was typically 5.8±0.36% (n=10 separate secretion assays).

For long-lasting catecholamine secretion, PC12 cells were treated with secretin (8 μM), and the supernatants were collected 20 min after treatment. Secretion buffer without secretin was added every 20 min followed by the collection of the supernatants after each subsequent 20-min period. At the end of the experiment (270 min), the cells were harvested and lysed for residual cellular norepinephrine content. Catecholamine secretion levels at 20 or 30 min time-points were evaluated by counting the amount of norepinephrine released and dividing by the sum of the amount released during that 20-min or 30-min period plus the amount remaining in the cells at the end of that time period.

Intracellular (endogenous) catecholamine levels were measured by using high performance liquid chromatography (HPLC) coupled to an electrochemical detector (Waters 600E Multisolvent Delivery system and Waters 2465 Electrochemical Detector, Mass., USA) as described previously (Gayen et al. 2009).

Biochemical measurements

Measurement of intracellular phospholipase C level—Intracellular phospholipase C (PLC) levels were measured as the ability of samples to release p-nitrophenol (pNP) from the chromogenic substrate p-nitrophenylphosphorylcholine (pNPPC) as described previously (Aragon et al. 2002; Kurioka and Matsuda 1976). Briefly, PC12 cells were treated with ascending doses of secretin (2–8 μM) or no secretin (control) for 30 s in secretion buffer. After aspiration of the buffer, cells were treated with lysis buffer for 20 min with shaking. The lysates were centrifuged at 1000g for 10 min, and 100 μl supernatant was added to 1 ml 50 mM HEPES (pH 7.5) buffer containing 5 mM $CaCl_2$, 5 mM $MnCl_2$, 3 mM sodium azide, 0.5% Triton X-100, and 2.5 mM pNPPC. After overnight incubation at 37°C, the amount of pNP was recorded by spectrophotometry at 410 nm, with 1 arbitrary unit (AU) of PLC activity being defined as that yielding 1 nmol pNP/h.

Measurement of intracellular inositol-triphosphate level—Intracellular inositol-1,4,5-triphosphate (IP_3) concentration was measured using the Biotrak D-*myo*-IP_3 assay system (GE Healthcare-Amersham Biosciences, Piscataway, N.J., USA). The amounts of IP_3 (in pmol/tube) in the samples were determined from a standard curve obtained by using purified D-*myo*-IP_3 at serially diluted concentrations.

Measurement of intracellular cAMP—Intracellular cAMP levels were measured by using an enzyme immunoassay (correlate-EIA direct cAMP kit; Assay Designs, Ann Arbor, MI., USA). The amounts of cAMP (in pmol/ml) in the samples were derived from the standards as described in the manufacturer's protocol.

Measurement of intracellular PKA activity—The level of intracellular PKA activity was measured by using a protein kinase assay kit (Calbiochem, San Diego, USA) based on enzyme-linked immunosorbent assay.

Immunoblot analysis of ERK and CREB

PC12 cells were grown to 80% confluency in 6-well tissue culture plates. Cells were stimulated in serum-free Dulbecco's modified Eagle's medium (DMEM) with and without 4 μM secretin for 0, 5, and 15 min. Cells were harvested in 250 μl 1×Laemmli reducing sample buffer, and 25 μl sample was resolved by NuPAGETM 4%–12% bis-TRIS SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 1.0 mm × 12-well gels (Invitrogen, Carlsbad, CA., USA). Phosphorylated-ERK (P-ERK; E-4 Sc 7383; Santa Cruz Biotechnology, Santa Cruz, CA., USA), total ERK (T-ERK; ERK 2 (C-14); Santa Cruz Biotechnology), and phosphorylated CREB (PKA phosphorylation site of CREB, P-CREB-Ser133; Cell Signaling Technology, Danvers, MA., USA) levels were assayed by immunoblot analysis as described by the manufacturer.

Electrophoretic mobility shift assays

Preparation of nuclear extracts—Saline- and 1 μM secretin-treated PC12 cells were grown in 10-cm-diameter tissue culture plates, and nuclear extracts were prepared by using a commercial kit (Cayman chemical, Ann Arbor, MI., USA). Protein concentrations were measured by using the Bio-Rad Coomassie-blue-dye-binding reagent (Bio-Rad Laboratories, Hercules, CA., USA).

Synthesis and labeling of oligonucleotides—Single-stranded oligonucleotides flanking the sequences of the *Th* promoter CREB consensus sequence (TGACGTCA) and their complementary (−) strands were synthesized and PAGE-purified by Valuegene

(San Diego, Calif., USA) at a concentration of 100 mM. The sequences were sense: rat TH _ CREB_ F : 5′ - GAGGGGCTTTGACGT-CAGCCTGGCCT-3′, rat_TH_CREB_R: 5′-AGGC-CAGGCTGACGTCAAAGCCCCTC −3. Each oligonucleotide (5 pmol) was labeled with biotin by the Biotin 3′ End DNA Labeling kit (Pierce, Rockford, Ill., USA). The oligonucleotides were annealed by mixing together equal amounts of labeled complementary oligonucleotides followed by incubation for 1 h at room temperature.

Assay—The binding was applied by means of the LightShift chemiluminescent EMSA kit (Pierce) in a 15-ml reaction complex, i.e., 1× binding buffer, 100 ng/µl Poly (dI.dC), with or without unlabeled target DNA (10 pmol), nuclear protein extract (4 mg), with or without CREB and pCREB antibodies (4 mg), and biotin-labeled oligonucleotide (50 fmol). After being incubated for 20 min at room temperature, the mixtures were loaded onto 5% non-denaturing polyacrylamide gels (5% acrylamide, 37.5:1 acrylamide: bisacrylamide) and run at 100 V in 0.5× TRIS-borate/EDTA buffer. The gels were transferred into nylon membranes (Pierce), and the biotin-labeled DNA was detected by chemiluminescence.

Chromatin immunoprecipitation assay

Immunoprecipitation assays were performed by using the chromatin immunoprecipitation (ChIP) assay kit from Upstate Biotechnology (Lake Placid, N.Y., USA) with slight modification. PC12 cells were treated with saline or secretin (1 µM) for 24 h. Chromatin from 5–10×10^6 cells was cross-linked in 1% formaldehyde for 10 min, washed in ice-cold PBS, and resuspended in SDS lysis buffer. After sonication and centrifugation, samples were pre-cleared with salmon sperm DNA and protein A agarose slurry followed by incubation with anti-CREB or p-CREB antibodies (Santa Cruz Biotechnology) at 4°C overnight. Immune complexes were captured with 30 ml protein A and salmon sperm DNA slurry and washed extensively with low salt, high salt, LiCl, and TRIS/EDTA buffer. DNA-protein complexes were eluted with 500 ml elution buffer at room temperature. Eluates were pooled with 20 ml 5 M NaCl and heated at 65°C for 4 h to reverse cross-links. DNA fragments were purified with QIAquick PCR purification kits (Qiagen, Valencia, Calif., USA). Rat-specific *Th* promoter primers (sense: 5′-agaggatgcgcaggaggtag-3′; anti-sense: 5′-gtcccgagttctgtctccac-3′) were used for PCR amplification over 15, 25, and 35 cycles to ensure that the 110-bp amplicons did not plateau in yield.

Peptides and chemicals

Human secretin (HSDGTFTSELSRLREGARLQRLLQGLV-amide) was purified to >97% homogeneity by reverse-phase HPLC and obtained either from Peninsula Laboratories (Belmont, Calif., USA) or from Calbiochem (San Diego, Calif., USA). Bepridil, ω-conotoxin GVIA, ω-agatoxin, ω-conotoxin MVIIC, BAPTA-AM, U-73122, U-73343, and 5-iodotubercidin were all obtained from Calbiochem. Nifedipine was obtained from Sigma (St. Louis, Mo., USA).

Data presentation and statistical analysis

The potency of secretin-induced catecholamine secretion and transcription was calculated from the determination of the EC_{50} (concentration required to give half-maximal effect in a Stineman smooth curve fit) value by using the program Kaleidagraph (Synergy/Abelbeck

Software, Reading, Pa., USA). Secretion and transfection experiments were repeated 2–3 times, with three wells per condition in each experiment. Results are expressed as the mean value ± one standard error of the mean of a representative experiment. Descriptive and inferential statistics were performed on representative experiments with the program InStat (GraphPad Software, San Diego, Calif., USA). Student t-tests or a one-way analysis of variance followed by Bonferroni's multiple comparison tests were used as appropriate. Significance was determined at the $P \leq 0.05$ level.

Results

Dose- and time-dependent effects on activation of Th gene expression

Secretin dose-dependently activated transcription of the *Th* gene as judged by the expression of secretin promoter-driven luciferase reporter activity with an EC_{50} of ~4.6 nM in PC12 cells (Fig. 1a). Activation of *Th* transcription was rapid, with an approximately six-fold increment as early as 3 h and attainment of the maximum (~24-fold) at 18 h after treatment (Fig. 1b). Real-time RT-PCR showed augmented transcription of the endogenous *Th* gene (by 2.1-fold) in response to secretin (Fig. 1c). To determine whether secretin acted through its own receptor (SCTR), we pre-treated cells with SCTR antagonist (secretin$_{5–27}$; 1 μM) 30 min before secretin; this abolished 0.1 μM secretin-induced transcription of the *Th* gene, suggesting secretin action through SCTR. Failure of PAC1 (PACAP$_{6–38}$) and VPAC1 (VIP$_{6–28}$) antagonists in the inhibition of secretin-induced transcription (Fig. 1d) indicates that secretin does not signal through these receptors.

Augmented cAMP production and PKA activity

Since cAMP levels are elevated in response to PACAP (Hamelink et al. 2002) and VIP (Anderova et al. 1998) in chromaffin cells, we reasoned that secretin would also increase intracellular cAMP levels. In agreement with this, we found dose-dependent increments in intracellular cAMP in response to secretin (Fig. 2a). The highest increment in cAMP levels (by ~327%; EC_{50}~0.2 μM) was observed at a dose of 2 μM secretin (Fig. 2a). This is consistent with the activation of PKA by secretin (Fig. 2b). We used PACAP as a positive control (Fig. 2b).

Transcriptional inhibition of Th gene after blockade of PKA activity

Consistent with the crucial role of the cAMP/PKA pathway in transcription of the genes of catecholamine storage vesicle proteins (Mahapatra et al. 2000, 2003; Mahata et al. 1999; Taupenot et al. 1998; Wu et al. 1995) and catecholamine biosynthetic enzyme genes (Choi et al. 1999; Corbitt et al. 2002), we found that a peptide inhibitor of PKA (expressed by RSV-PKI) caused substantial inhibition (by ~88%–92%) of secretin-induced transcription of the *Th* gene (Fig. 2c). Likewise, chemical blockade of PKA by H-89 caused substantial inhibition (by ~56%–64%) of *Th* promoter activity in response to secretin (Fig. 2d).

Mitogen-activated protein kinase pathway in regulation of transcription of the Th gene

Chemical blockade of ERK by 5-iodotubercidin (0.1–2 μM) caused profound inhibition (by ~90%) of transcription of *Th* gene induced by secretin (Fig. 2e). Consistent with the biochemical findings, we found a dramatic increase in the phosphorylation of ERK (Fig. 2f) in response to secretin.

Regulation of transcription of Th gene by CREB

PC12 cells transfected with the *Th* promoter/reporter plasmids were co-transfected with a dominant negative mutant of CREB (KCREB); this caused substantial diminution (by ~86%) of *Th* gene transcription induced by secretin (Fig. 3a), indicating secretin signaling via CREB. This finding is supported by secretin-induced phosphorylation of CREB (P-CREB-Ser133) (Fig. 3B).

Augmentation phosphorylation of CREB and its binding to Th-CRE

Secretin augmented binding of PC12 nuclear proteins to the *Th*-CRE oligomer, suggesting involvement of the CREB/ATF transcription factor family (Fig. 3c, lanes 1, 5). To confirm the binding of a CREB/ATF protein to the CRE region, we performed supershift assays with an antibody that specifically recognizes CREB and does not cross-react with other ATF/CREB proteins (Fig. 3c, lanes 3, 7). The higher binding activity of CREB in the presence of secretin is shown by the supershift of the major complex to a greater extent as compared with the saline-treated sample (Fig. 3c, lanes 3, 7). Because CRE-dependent *trans*activation requires the phosphorylation of CREB at serine 133, we also performed supershift assays with an antibody directed against the serine-133-phosphorylated CREB. Although secretin caused marked shifting of the phosphorylated CREB-CRE complex, no supershift was observed with this antibody (Fig. 3c, lanes 4, 8).

Increased in vivo binding of pCREB to the endogenous Th promoter

In saline-treated cells, both CREB and pCREB are captured by ChIP, with the intensity of CREB being much stronger than that of pCREB. In secretin-treated cells, only pCREB is captured by ChIP with strong intensity (Fig. 3d) confirming the increased in vivo binding of pCREB to the endogenous *Th* promoter in response to secretin.

Dose-dependent effects on catecholamine secretion

Secretin evoked dose-dependent catecholamine secretion (EC_{50} ~3.5 μM) in PC12 cells (Fig. 4a). The maximal (ceiling) effect (up to 26% of cell stores) was seen at 10 μM, which then declined to 20 μM, suggesting the desensitization of the SCTR at the highest agonist dose. Secretin also evoked catecholamine secretion from the endogenous stores in PC12 cells (Fig. 4b). Of note, catecholamine secretion in response to secretin alone was almost identical to the cumulative effects of all three agonists of the superfamily (secretin plus PACAP plus VIP; Fig. 4c) indicating that all three agonists share the same receptor in triggering the secretory response, a conclusion consistent with the antagonist results (Fig. 4d). Unlike transcription, SCTR antagonist (even at 20 μM) failed to inhibit secretion (Fig. 4d). Secretion, however, was inhibited by pre-treatments with the receptor antagonists VPAC1 ($VIP_{6–28}$: IC_{50} >20 μM) or PAC1 ($PACAP_{6–38}$: IC_{50} ~11.3 μM) (Fig. 4d) indicating secretin signaling to the catecholamine secretion pathway through VPAC1/PAC1 receptors.

As opposed to transcription, we found a maximal effect of secretin on catecholamine secretion at 10-μM doses and a significant effect at the 1-μM dose. Thus, secretin exerts its effect on secretion on a narrow window (1–10 μM). We detected a similar effect with PACAP (Taupenot et al. 1998). Recently, analogous effects were shown for serpinin (Koshimizu et al. 2011). Therefore, we tested several concentrations of secretin and report the secretin dose that gave the maximal effect in each experiment. The receptors for secretin, VIP, and PACAP are SCTR (secretin-preferring), VPAC1 (VIP-preferring), and PAC1 (PACAP-preferring), respectively. The commercially available antagonists are not very specific. Therefore, any definite conclusion concerning the involvement of the receptors for secretin, VIP, and PACAP are difficult to make.

Lack of involvement of plasma membrane Ca^{2+} channels in catecholamine secretion

Catecholamine secretion depends on increments in cytosolic Ca^{2+} concentrations resulting through either an influx from the extracellular space or a release from intracellular stores (Douglas 1968; Garcia et al. 2006; Grabner and Fox 2006). Our initial studies with chemical inhibitors of plasma membrane Ca^{2+} channels yielded little to no effect on secretin-induced catecholamine secretion. We used the following inhibitors of Ca^{2+} channels to determine the involvement of sub-type(s)-specific Ca^{2+} channels: L-type (nifedipine, 10 μM), N-type (ω-conotoxin GVIA, 1 μM), P-type (ω-agatoxin, 0.5 μM), Q-type (ω-conotoxin MVIIC, 0.5 μM), or T-type (bepridil, 10 μM) Ca^{2+} channels (Fig. 5a). In addition, non-specific blockade of plasma membrane Ca^{2+} channels by a divalent metal cation ($ZnCl_2$, 100 μM; Taupenot et al. 1998) was also ineffective on catecholamine secretion induced by secretin (Fig. 5a). As a positive control, PACAP-induced catecholamine secretion was partially (57%) or completely blocked by nifedipine (10 μM) or $ZnCl_2$ (100 μM), respectively (data not shown).

Catecholamine secretion in absence of extracellular Ca^{2+}, and its blockade in the presence of the intracellular Ca^{2+} chelator BAPTA-AM

Based on the above findings, we tested secretin-evoked catecholamine secretion in the presence or absence of extracellular Ca^{2+} (Fig. 5b) where Ca^{2+}-free buffer was supplemented with EGTA (0.5 mM). Catecholamine secretion in response to secretin was not affected by removal of extracellular Ca^{2+} (Fig. 5b).

Subsequently, we determined that pre-treatment of PC12 cells with a known cytosolic Ca^{2+} chelator, BAPTA-AM (50 μM), completely abolished catecholamine secretion induced by secretin (Fig. 5c). This indicated the crucial role played by Ca^{2+} release from intracellular stores in secretin signaling to catecholamine secretion.

PLC modulation of catecholamine secretion

The main pathway for Ca^{2+} release from intracellular sites is believed to be through the action of IP_3 on its receptor at the endoplasmic reticulum membrane. Activation of PLC-β by monomeric α-subunits of the G_q subfamily of Gα subunits generates IP_3 through poly-phosphoinositide hydrolysis. In the present study, PLC activity was increased in response to secretin (Fig. 6a), as supported by the observation that chemical inhibition of PLC-β (U73122, 10 μM) markedly (~54%) inhibited secretin-evoked catecholamine secretion (Fig. 6b) and indicating the involvement of PLC/IP_3 in this process. As a negative control, the inactive isomer (U-73343, 10 μM) of U-73122 did not affect secretin-induced secretion (Fig. 6b).

Increased IP_3 levels

Consistent with phospholipase inhibition, we found dose-dependent increments in IP_3 levels (by up to ~207% at 8 μM dose; EC_{50} ~1.6 μM) in response to secretin (Fig. 6c); this was further supported by the marked (~61%) inhibition of IP_3 levels after chemical blockade of phospholipase C with U73122 (20 μM; Fig. 6d).

Prolonged catecholamine secretion

Consistent with PACAP findings (Taupenot et al. 1999), we found that secretin (8 μM) induced long-lasting catecholamine secretion (up to 270 min) after an acute exposure of just 20 min (Fig. 7).

Discussion

The present findings indicate that secretin induces catecholamine biosynthesis by augmenting transcription of the *Th* gene. The circulating concentration of secretin is typically measured at ~10 nM (O'Donohue et al. 1981), and plasma levels of the peptide are shown to increase significantly in diabetes mellitus (Trimble et al. 1977). Secretin activates the expression of both the endogenous *Th* gene (Fig. 1c) and its transfected promoter (EC_{50} ~4.6 nM; Fig. 1a). Of note, secretin activates *Chga* gene transcription with an EC_{50} ~7 nM (Mahapatra et al. 2003). The results are relevant to the regulation of human blood pressure, since polymorphisms in the proximal promoters of the *Th* gene are associated with the development of hypertension (Cui et al. 2003; Rao et al. 2007). Other groups have reported an increase in *Th* gene expression (by ~250%; Wessels-Reiker et al. 1993) in addition to increments in TH activity in PC12 cells (Roskoski et al. 1989) and in sympathetic neurons (Ip and Zigmond 2000). Consistent with our data, secretin has been reported to be ineffective in increasing *Th* mRNA levels in a PKA-deficient cell line (A126–1B2) or in presence of adenylate cyclase inhibitor (Wessels-Reiker et al. 1993). Here, we show, for the first time, that secretin signaling to *Th* gene expression requires the activation of ERK and CREB.

The blockade of secretin-induced transcription of *Th* gene by a SCTR antagonist (Fig. 1d) and not by PAC1 or VPAC1 antagonists indicates secretin signaling through its own receptor for a transcriptional effect. SCTR antagonist has been reported to have no significant effect on VIP or peptide histidine isoleucine (PHI)-induced activation of *Th* gene expression but reduces the effect of secretin (Wessels-Reiker et al. 1993). Wessels-Reiker et al. (1993) have also found that the VPAC1 antagonist lowers the effect of VIP on increasing *Th* mRNA but exerts no significant effect on the *Th* gene expression induced by secretin or PHI. A diminution of secretin-induced *Th* gene transcription by the overexpression of PKI in PC12 cells (Fig. 2c) or by chemical blockade of PKA (Fig. 2d) and an increase in PKA activity (Fig. 2b) in response to secretin suggest that secretin acts through the cAMP-PKA pathway to induce *Th* gene transcription. The ultimate target of PKA is the transcription factor CREB (Shaywitz and Greenberg 1999). Following increases in intracellular cAMP levels and the activation of PKA, the catalytic subunit of PKA translocates into the nucleus and phosphorylates CREB at Ser-133, leading to the stimulation of gene transcription (Hagiwara et al. 1993). Subsequently, additional CREB kinases have been identified, including members of the calcium/calmodulin-dependent kinase family (Deisseroth et al. 1996; Sun et al. 1994) and the ERK-stimulated RSK kinases (Deak et al. 1998; Xing et al. 1996). Consistent with the above literature, we have found that chemical inhibition of ERK (Fig. 2e) or overexpression of a dominant negative mutant of CREB (Fig. 3a) almost completely abolishes secretin-induced transcription of the *Th* gene. Dramatic increments in the phosphorylation of ERK (Fig. 2f) and CREB (Fig. 3b) in response to secretin reinforce the above findings. Of note, secretin induces *Chga* gene transcription through the CRE domain in cis and through cAMP, PKA, mitogen-activated protein kinase, and the transcription factor CREB in *trans* (Mahapatra et al. 2003). Activation of CREB has been reported to induce *Th* gene transcription (Lazaroff et al. 1995; Lewis-Tuffin et al. 2004; Tinti et al. 1997). Although we have documented the effects of secretin on the steady-state level of *Th* transcripts in the chromaffin cell (Fig. 1c), we have not directly examined the rate of initiation of new transcripts or the stability of *Th* mRNA.

The present findings reveal that secretin at micromolar concentrations is a novel secretagogue of catecholamine secretion (EC_{50}~3.5 μM, Fig. 4a), joining other peptidergic chromaffin cell secretagogues from the PACAP/VIP/secretin family: PACAP and VIP (Taupenot et al. 1998). Amongst the peptidergic secretagogs, PACAP is the most potent with an EC_{50} of ~12 nM (Taupenot et al. 1998), followed by VIP (Guo and Wakade 1994; Wakade et al. 1991) and secretin (EC_{50} ~3.5 μM). Significant catecholamine secretion is achieved by 10 nM PACAP (Chowdhury et al. 1994; Przywara et al. 1996), 300 nM VIP (Guo and Wakade 1994; Wakade et al. 1991), and 1000 nM secretin (present study). These findings indicate that secretin and VIP are poor agonists for the PAC1 receptor. Our results might be pertinent to the known pharmacological effects of higher dose of secretin. Thus, secretin at pharmacological doses has been reported to enhance left ventricular function in the intact anesthetized dog by combined vasodilating, inotropic, and chronotropic effects, without changing myocardial oxygen or substrate uptake (Gunnes et al. 1985). In addition, in patients with depressed cardiac function or in the closed-chest dog model of acute ischemic left ventricular failure, pharmacological doses of secretin are reported to increase left ventricular performance by means of arteriolar dilation and a positive inotropic effect (Gunnes and Rasmussen 1986; Gunnes et al. 1986). We should also mention that the plasma levels of secretin are increased in diabetes mellitus (Trimble et al. 1977), and that diabetes modulates the cardiovascular action of secretin (Chatelain et al. 1983; Sitniewska et al. 2002). Thus, our results concerning catecholamine secretion might also be pertinent to pathophysiological/pharmacological concentrations of the peptide.

Increments in cytosolic Ca^{2+} are a prerequisite for catecholamine secretion, achieved through either an influx of Ca^{2+} from the extracellular medium or the release from intracellular stores (Douglas 1968; Garcia et al. 2006; Grabner and Fox 2006). Since chemical inhibitors of plasma membrane Ca^{2+} channels (including L, N, P, Q, or T types) and the non-specific blockade of calcium channels by $ZnCl_2$ do not reduce stimulated catecholamine secretion (Fig. 5a), we conclude that Ca^{2+} release from intracellular stores must be crucial for this process. This conclusion is supported by the findings that secretin-induced catecholamine secretion occurs in the absence of extracellular Ca^{2+} (Fig. 5b), and that secretion is blocked by the intracellular Ca^{2+}-chelator, BAPTA-AM (Fig. 5c). The signaling pathway that mediates secretin-induced catecholamine secretion is in congruence with VIP where catecholamine secretion depends solely upon Ca^{2+} mobilization from intracellular stores (Malhotra et al. 1988). In contrast, PACAP-induced catecholamine secretion is markedly/almost completely inhibited by chemical inhibition of the following: non-specific Ca^{2+} channel by $ZnCl_2$, L-type specific Ca^{2+} channel by nifedipine, Ca^{2+} release from ryanodine-sensitive intracellular Ca^{2+} stores by ruthenium red, or chelation of intracellular Ca^{2+} by BAPTA-AM (Przywara et al. 1996; Taupenot et al. 1998). Therefore, PACAP utilizes both Ca^{2+} uptake from the extracellular space and Ca^{2+} release from intracellular stores to evoke catecholamine secretion. In contrast, secretin-induced catecholamine secretion is dependent on Ca^{2+}, which comes exclusively from the intracellular stores.

Low concentrations of secretin act through a $G\alpha_s$ → adenylyl cyclase → cAMP pathway (Mahapatra et al. 2003). In contrast to secretin, low doses of PACAP trigger a $G\alpha_q$ → phospholipase C-β (PLC-β) → IP_3 → intracellular Ca^{2+} signaling pathway (Taupenot et al. 1998). PLC-β is activated by monomeric α-G-protein subunits of the G_q family or the βγ heterodimeric subunits of the $G_{i/o}$ G proteins to generate IP_3 through the hydrolysis of polyphosphoinositides. IP_3 acts on its receptor to release Ca^{2+} stored inside the endoplasmic reticulum. Stimulation of bradykinin membrane G-protein-coupled receptors (GPCRs) coupled to IP_3 formation and Ca^{2+} release from intracellular stores can cause catecholamine secretion (Berridge 1998). Consistent with the above pathways, we have found acute (15 s) increases in IP_3 levels (by ~207%) in response to secretin (Fig. 6c). The role of such a pathway is reinforced by secretin increasing PLC activity (Fig. 6a) and by substantial (by ~54%) inhibition of secretin-mediated catecholamine secretion (Fig. 6b) by U-73122, which is a specific inhibitor of PLC-β (Fig. 6d). Based on these findings, we propose that the GPCR of secretin is likely to signal to catecholamine secretion through G_q/G_{11} α-subunits to activate PLC-β, which in turn induces the formation of IP_3, which releases Ca^{2+} from intracellular stores. Of note, the cAMP response to secretin is substantially more pronounced than the IP_3 response (Figs 2a, 6c) both in magnitude (stimulation of up to approximately three-fold of basal) and potency (near-maximal stimulation at submicromolar dose); the lower response of the cAMP versus the IP_3 pathway to secretin is consistent with the lower potency of secretin on secretion versus transcription (Mahapatra et al. 2003) in chromaffin cells.

Like PACAP (Taupenot et al. 1999), secretin also induces long-lasting (at least up to 270 min) catecholamine secretion from PC12 cells, even after the removal of the original peptide stimulus (Fig. 7). This long-lasting PACAP/VIP/secretin family peptidergic response is likely to represent exocytosis, since the blockade of cell-surface calcium channels prevents prolonged secretion (Taupenot et al. 1999). Long-lasting catecholamine secretion by PACAP and secretin might have physiological implications as is evident from studies in mice with a targeted deletion of the PACAP gene (Hamelink et al. 2002). PACAP-deficient mice fail to counter-regulate low plasma glucose levels adequately because of an impairment of the long-term secretion of epinephrine (Hamelink et al. 2002). These findings indicate a critical role for the PACAP/VIP/secretin superfamily in the maintenance of adrenomedullary epinephrine secretion in the face of prolonged metabolic stress (Hamelink et al. 2002). In sharp contrast to peptidergic secretagogues such as PACAP and secretin, nicotine does not cause prolonged catecholamine secretion (Hamelink et al. 2002). We therefore suggest that, like PACAP (Taupenot et al. 1999), secretin might also play a pivotal role in counteracting prolonged metabolic stress by evoking long-lasting catecholamine secretion.

In summary, we report several lines of evidence that confirm that secretin acts with markedly different concentration requirements to trigger transcription (nanomolar) versus secretion (micromolar). Only nanomolar concentrations of secretin induce transcription (*Chga*: EC_{50} ~7 nM; Mahapatra et al. 2003; *Th*: EC_{50} ~4.6 nM) and micromolar concentrations of the peptide are needed to stimulate catecholamine secretion (Fig. 4a; EC_{50} ~3.5 μM). Our studies also show that these two processes are likely to be mediated by different receptors and signal transduction pathways. Secretin-induced transcription is mediated by the activation of the SCTR → $G\alpha_s$ → adenylyl cyclase → cAMP → PKA/ERK → CREB pathway. In contrast, secretin-induced catecholamine secretion is mediated by the activation of the PAC1/VPAC1 receptors → $G\alpha_q/G\alpha_{11}$ → phospholipase C-β→ IP_3 → intracellular Ca^{2+} pathway. Our secretory and transcriptional results are represented in Fig. 8.

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Abbreviations

Chga	Chromogranin A gene
CRE	cAMP response element
ERK	Extracellular-signal-regulated kinase
GPCR	G-protein-coupled receptor
IP_3	Inositol-1,4,5-triphosphate
PAC1	PACAP-preferring receptor
PACAP	Pituitary adenylyl cyclase activating polypeptide
PHI	Peptide histidine isoleucine
PKA	Protein kinase A
PLC	Phospholipase C
RT-PCR	Reverse transcription followed by polymerase Chain reaction
SCTR	Secretin receptor
Sctr	Secretin receptor gene
TH	Tyrosine hydroxylase enzyme
Th	TH gene
VIP	Vasoactive intestinal polypeptide

VPAC1	VIP-preferring receptor

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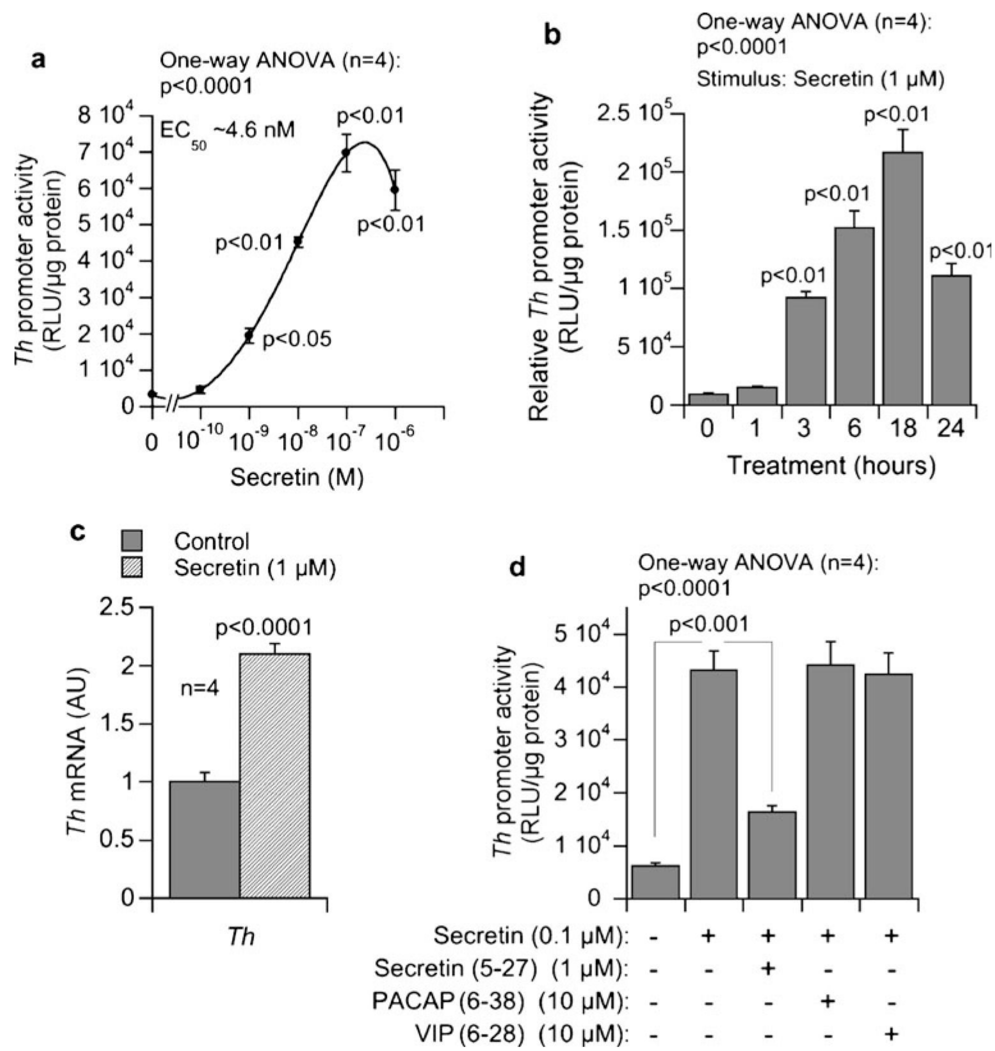

Fig. 1.

Transactivation of the *tyrosine hydroxylase* (*Th*) promoter and activation of the endogenous *Th* gene by secretin in PC12 cells. **a** Dose-dependent effects. PC12 cells transfected with rat *Th* (4.8 kb promoter/luciferase reporter) promoter were treated with logarithmically ascending doses (0.0001–1 µM) of secretin for 18 h. Cells were then harvested for luciferase activity assay and cell protein concentration. Results are expressed as relative light units (*RLU*) normalized to microgram cell protein (*ANOVA* analysis of variance, EC_{50} concentration required to give half-maximal effect in a Stineman smooth curve fit). **b** Time-dependent effects. PC12 cells transfected with rat *Th* promoter were treated with secretin (1 µM) for 24 h, 18 h, 6 h, 3 h, and 1 h before harvesting for measurement of luciferase activity and cellular protein. **c** Expression of the endogenous *Th* gene. RNA was extracted from secretin-treated (1 µM for 18 h) and control PC12 cells, and expression of the gene was analyzed by real-time reverse transcription followed by the polymerase chain reaction. **d** Blockade of transcription by secretin receptor (SCTR) antagonist. Transfected cells were treated with control, secretin (0.1 µM), or SCTR antagonist (secretin$_{5–27}$; 1 µM) plus secretin (0.1 μM) for 18 h before harvesting for luciferase and protein assays (*PACAP* pituitary adenylyl cyclase activating polypeptide, *VIP* vasoactive intestinal polypeptide)

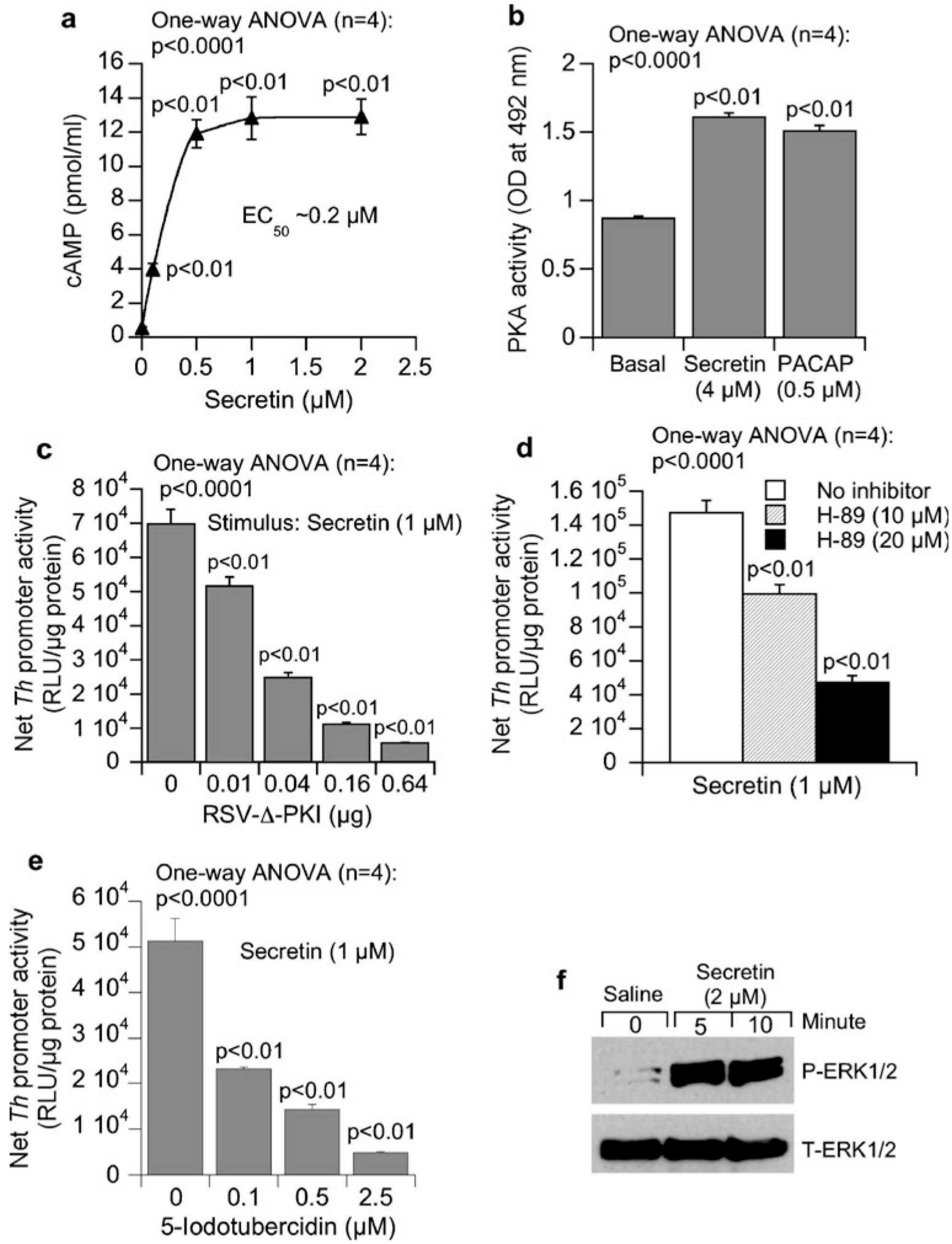

Fig. 2.

Effect of secretin signaling on *Th* gene transcription. **a** Intracellular cAMP level. PC12 cells were treated with ascending doses of secretin (0.1–8 μM) or no secretin (control) for 0.5 min, and the cell extracts were subjected to cAMP assay (see Materials and methods). The results are shown as the cAMP level (pmol/ml). **b** Protein kinase A (*PKA*) activity. PC12 cells were treated with secretin (4 μM) or PACAP (0.5 μM), and PKA activity was measured by an enzyme immunoassay (see Materials and methods). **c** Overexpression of a PKA inhibitor plasmid. PC12 cells were transfected with a *Th* promoter/luciferase reporter construct and co-transfected with the PKA inhibitor plasmid (*PKI*). Co-transfected cells were treated with secretin (1 μM) or no secretin (control) for 18 h before being harvested for luciferase assay. **d** Chemical inhibitor of PKA. Cells transfected with *Th* promoter 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 being harvested for luciferase assay. **e** Chemical inhibitor of extracellular-signal-regulated kinase (ERK). Cells transfected with *Th* were treated with chemical inhibitors of ERK (5-iodotubercidin, 0.1–2 μM) either alone or in combination with secretin (1 μM) for 18 h before being harvested for luciferase assay. **f** ERK immunoblot. An immunoblot for phosphorylated ERK (*P-ERK*) and total ERK (*T-ERK*) from total cell lysates of PC12 cells stimulated with 2 μM secretin for 0, 5, and 15 min

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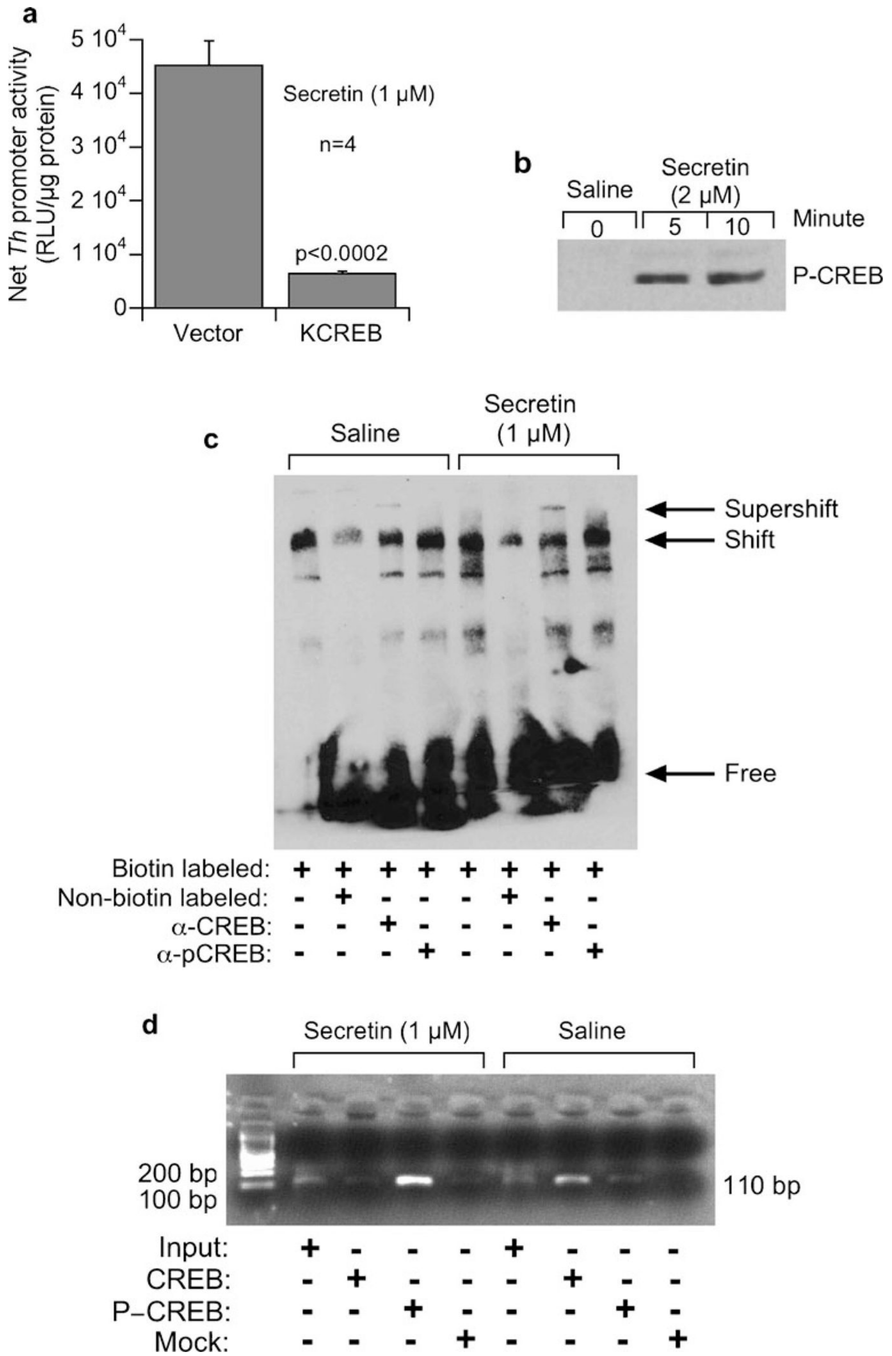

Fig. 3.

Regulation of *Th* gene transcription by CRE-binding protein (CREB). a Overexpression of **a** CREB inhibitor plasmid. PC12 cells were transfected with a *Th* promoter/luciferase reporter construct and co-transfected with the CREB inhibitor plasmid (*KCREB*). Co-transfected cells were treated with secretin (1 µM) or no secretin (control) for 18 h before being harvested for luciferase assay. **b** Phosphorylation of CREB at Ser-133. Immunoblot analysis of P-CREB-133 levels in PC12 cells stimulated with 2 µM secretin for 0, 5, and 15 min. **c** Electrophoretic mobility supershift assay. Nuclear proteins were extracted from mock-versus secretin (1 μM)-treated PC12 cells, which were incubated with the labeled double-stranded *Th*-CRE oligonucleotide probe either alone or in the presence of anti-CREB (*α-CREB*) or anti-phospho-CREB (*α-pCREB*). **d** Endogenous CREB motif and chromatin immunoprecipitation (ChIP); *lane 1* DNA size-standards (100-bp DNA ladder), *lanes 2, 5* Input, which represents 100-times less fragmented DNA than other samples but without immunoprecipitation (*Input*), *lanes 3, 6* chromatin immunoprecipitation by anti-CREB antiserum (CREB), *lanes 4, 7* chromatin immunoprecipitation by anti-phospho-CREB antiserum (*P-CREB*), lanes 5, 8 mock, which represents the same amount of fragmented DNA as the CREB or pCREB sample but immunoprecipitated by non-specific IgG antiserum (*Mock*)

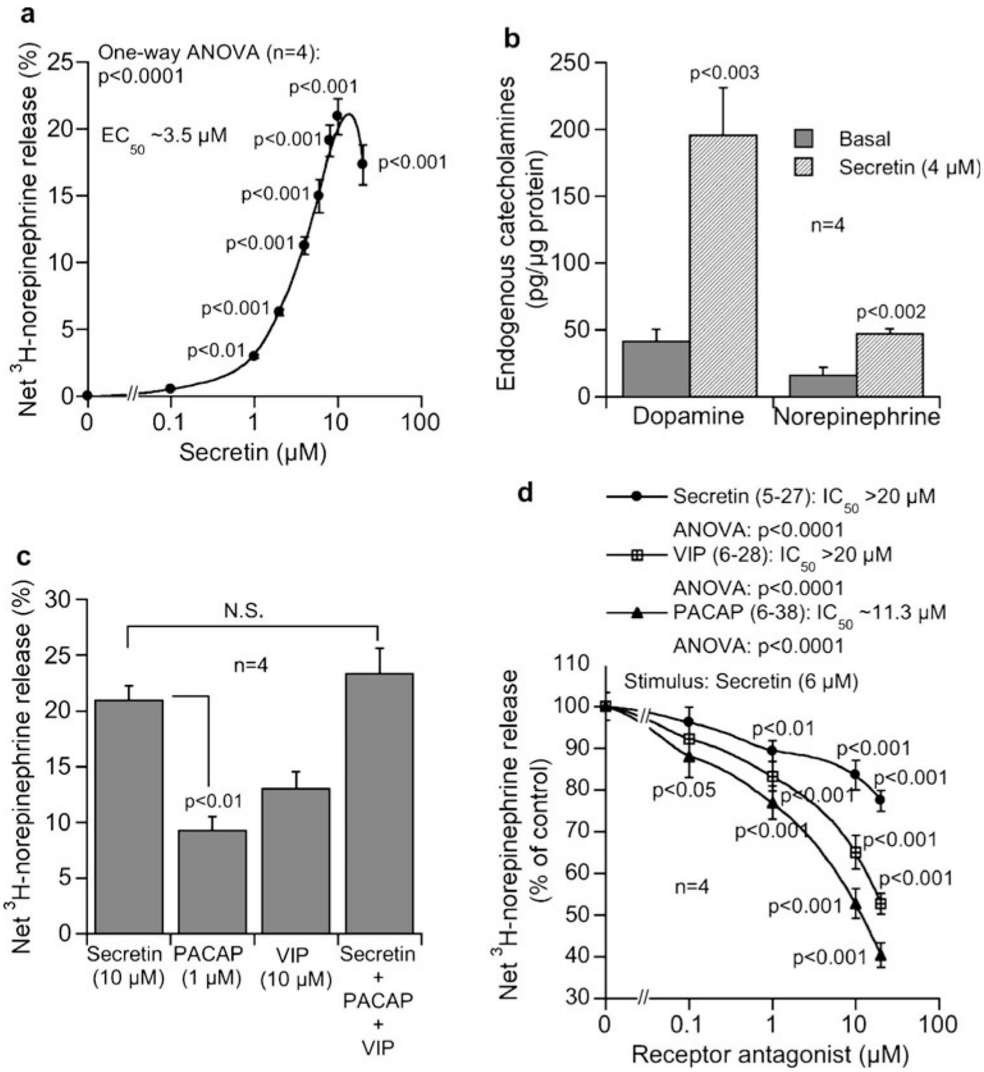

Fig. 4.

Acute effects of secretin on catecholamine secretion. **a** Dose-response study. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were treated with ascending doses of secretin (0.1–20 μM) for 20 min for measurement of norepinephrine secretion (*EC_{50}* concentration required for half-maximal stimulation of catecholamine release). **b** Endogenous catecholamine levels. Secretin increases dopamine and norepinephrine levels as measured by HPLC. **c** Individual versus cumulative effects of the peptides. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were treated with secretin (10 μM), PACAP (1 μM), VIP (10 μM), or a combination of secretin, PACAP, and VIP for 20 min for measurement of norepinephrine secretion. **d** Receptor antagonists. [^{3}H]-L-norepinephrine-prelabeled PC12 cells were incubated with secretin (6 μM), either alone or in combination with ascending doses (0.1–20 μM) of SCTR (secretin$_{5–27}$), VPAC1 (VIP$_{6–28}$) or PAC1 (PACAP$_{6–38}$) antagonists for 30 min. Each data point represents mean data from three separate wells. Control (100%) net norepinephrine release is that released in the presence of secretin (6 μM) stimulation alone, without antagonists

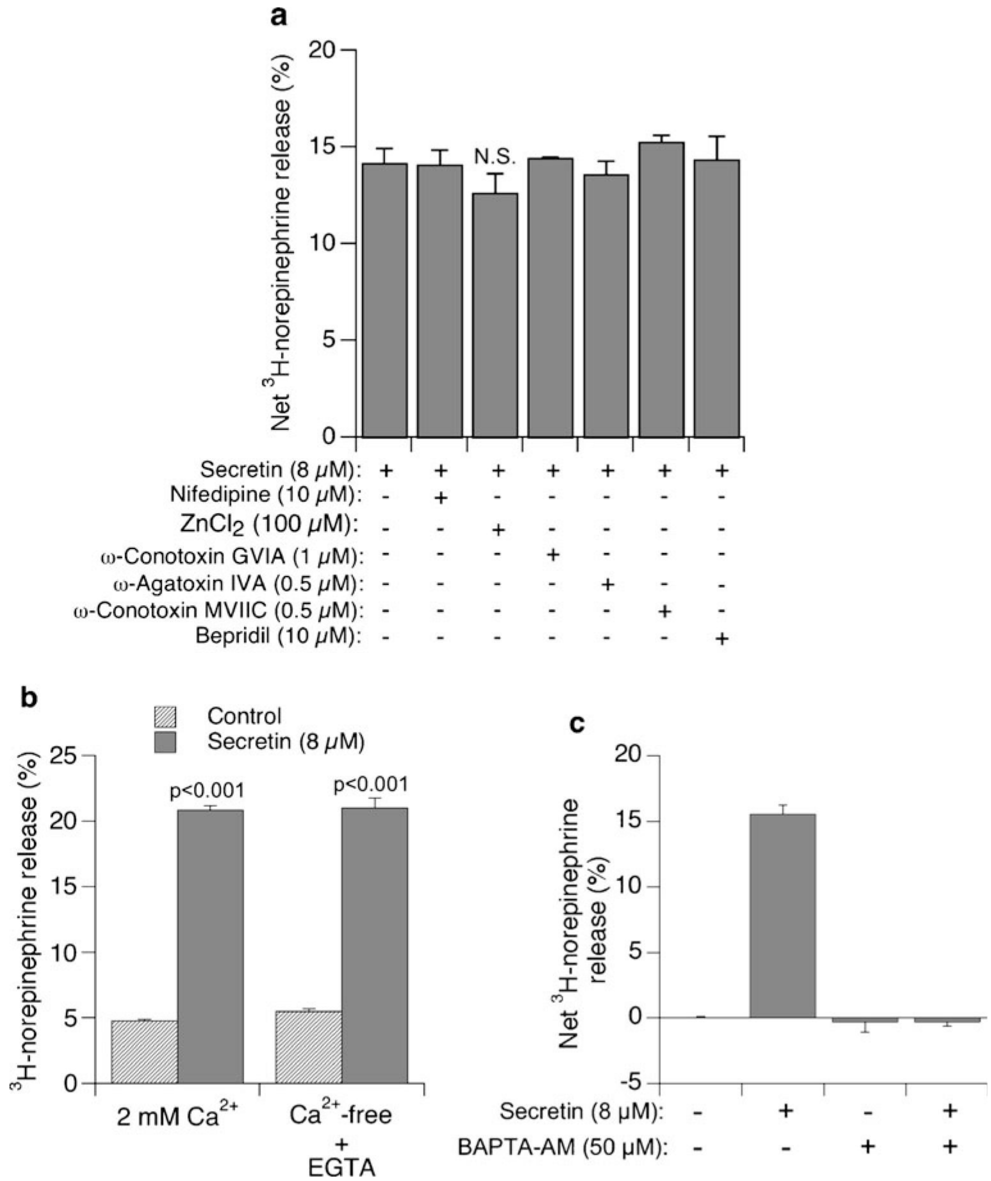

Fig. 5.

Role of extra- and intracellular Ca^{2+} in secretion. **a** Ca^{2+} influx from extracellular medium. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were treated with secretin (8 μM), either alone or in combination with nifedipine (10 μM), $ZnCl_2$ (100 μM), ω-conotoxin GVIA (1 μM), ω-agatoxin IVA (0.5 μM), ω-conotoxin MVIIC (0.5 μM), or bepridil (10 μM) for 20 min for measurement of norepinephrine secretion. **b** Extracellular Ca^{2+}. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were treated with secretin (8 μM) either in the presence or absence of extracellular Ca^{2+} for 20 min for the measurement of norepinephrine secretion. When extracellular calcium was absent, 0.5 mM EGTA was present. **c** Intracellular Ca^{2+}. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were exposed to Ca^{2+}-free buffer, in the presence or absence of pre-treatment (30 min) with the intracellular Ca^{2+} chelator BAPTA-AM (50 μM). Ca^{2+}-free buffer included 0.5 mM EGTA. Pre-treated cells were then treated with secretin (8 μM), BAPTA-AM (50 μM), or secretin plus BAPTA-AM in Ca^{2+}-free buffer plus EGTA (0.5 mM) for 20 min for measurement of norepinephrine secretion

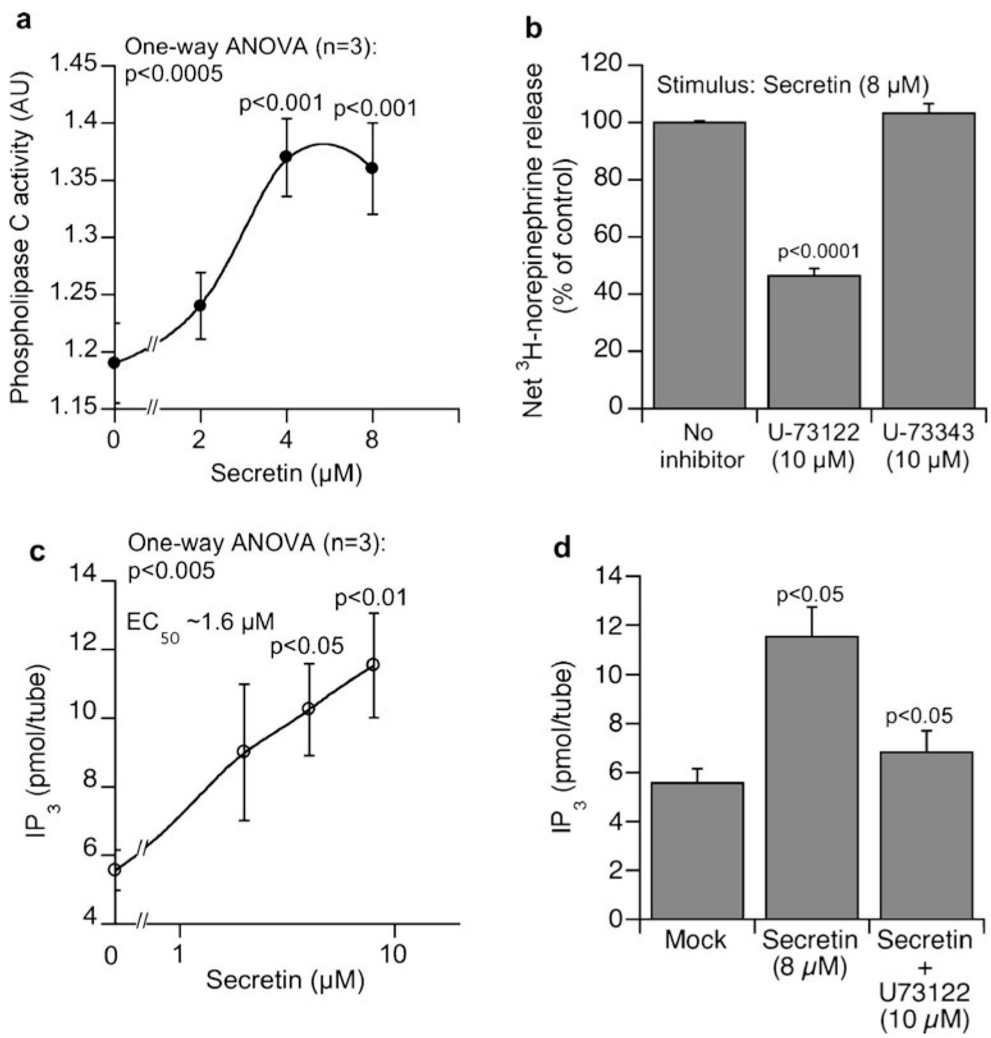

Fig. 6.

Effects of secretin on phospholipase C-β (PLC-β), inositol-1,4,5-triphosphate (*IP_3*) and cAMP. **a** Augmentation of PLC activity. PC12 cells were treated with secretin (0, 2, 4, or 8 μM) for 30 s before being harvested for PLC assay. **b** Blockade of PLC-β. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were treated with secretin (8 μM) either alone or in combination with the PLC-β inhibitor U-73122 (10 μM) or its inactive analog U-73343 (10 μM) for 20 min for measurement of norepinephrine secretion. Control (100%) net norepinephrine release is that release caused by secretin (8 μM) alone, without any inhibitor. **c** Generation of IP_3. PC12 cells were treated with ascending doses of secretin (0.5–8 μM) or no secretin (control) for 0.5 min, and the cell extracts were subjected to IP_3 assay (see Materials and methods). The results are shown as the IP_3 level (pmol/well). **d** Blockade of generation of IP_3. PC12 cells were treated with secretin (8 μM) either alone or in combination with the PLC-β inhibitor U-73122 (20 μM) for 0.5 min, and the cell extracts were subjected to IP_3 assay (see Materials and methods). The results are shown as the IP_3 level (pmol/well)

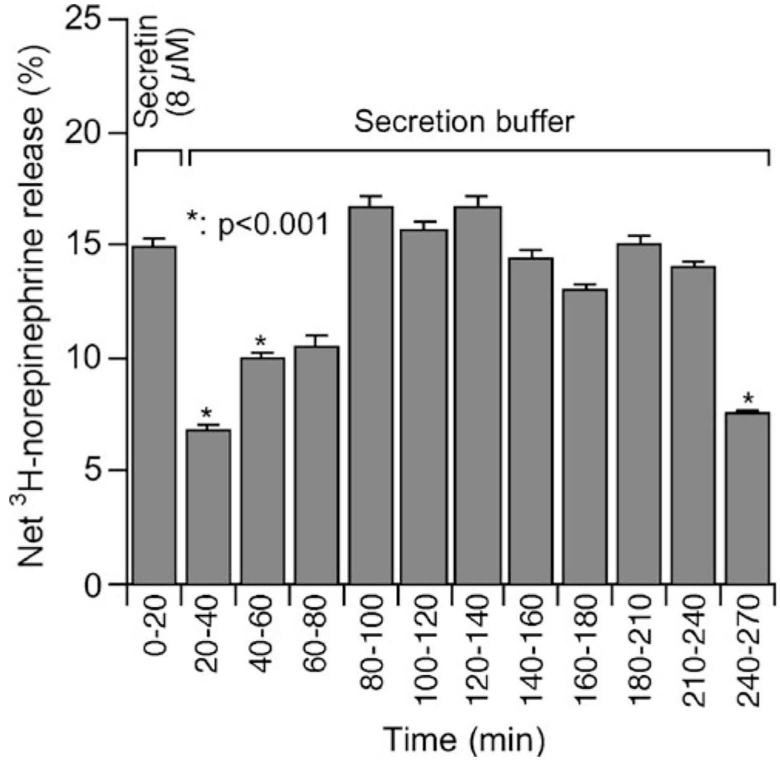

Fig. 7.

Prolonged catecholamine secretion in response to secretin. L-[^{3}H]-norepinephrine-prelabeled PC12 cells were treated with secretin (8 μM) for 20 min for measurement of norepinephrine secretion. After a 20-min incubation, extracellular media were collected and replaced by secretion buffer alone, followed by further incubations for the indicated time periods. At the final 30-min time-point, cells were lysed to determine cellular L-[^{3}H]-norepinephrine content. Catecholamine secretion at any 20-min or 30-min time-point was evaluated by counting the amount of norepinephrine released and dividing by the sum of the amount released during that 20 or 30 min plus the amount remaining in the cells at the end of that time-period. *P*-values: comparison with the “0–20” min group

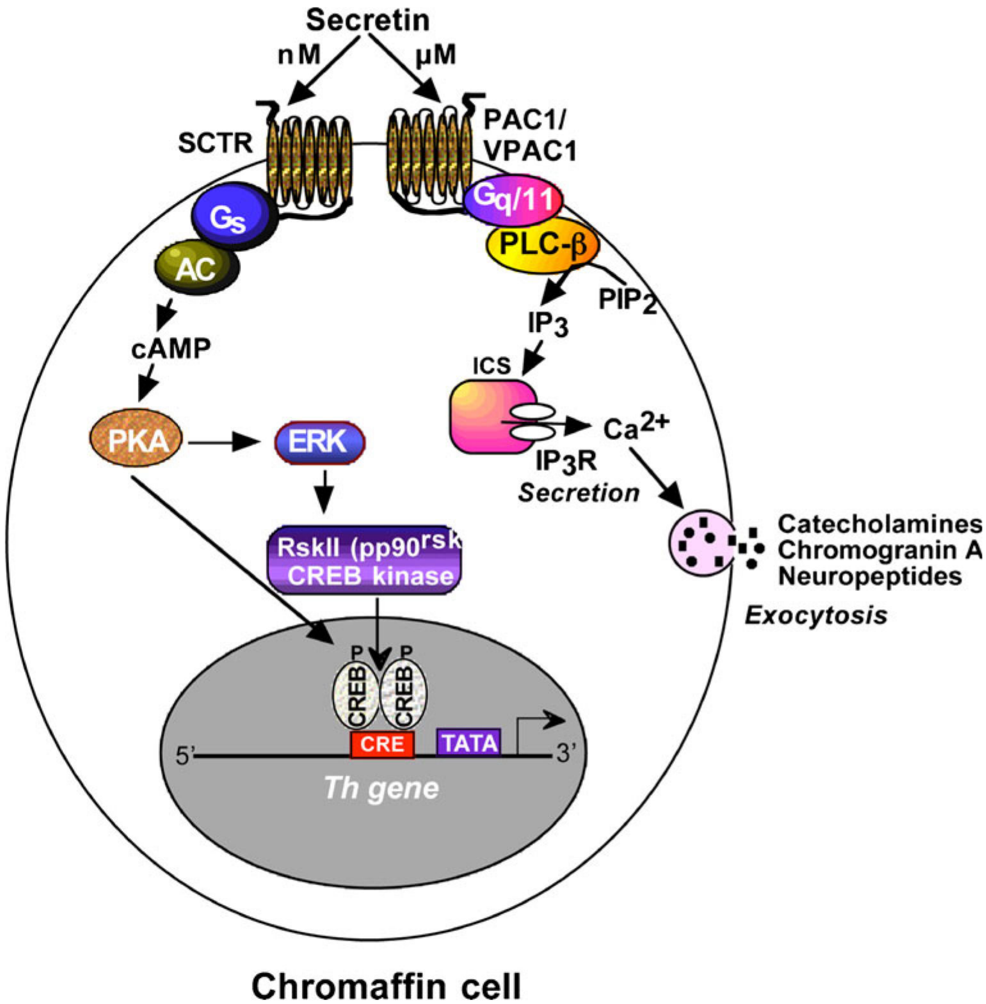

Fig. 8.

Representation of proposed signal transduction for secretin activation of *Th* gene transcription and catecholamine secretion, as suggested by the results obtained in the current experiments (*AC* adenylyl cyclase, *CRE* cAMP response element, *CREB* homodimeric CRE-binding/trans-activating protein, *ERK* mitogen-activated protein kinase/extracellular-signal-regulated kinase, G_s stimulatory heterotrimeric G-protein, $G_{q/11}$ heterotrimeric G-protein of the $G_{q/11}$ family, ICS intracellular Ca^{2+} store, IP_3 inositol-1,4,5-triphosphate, IP_3R receptor for IP_3, PIP_2 phosphatidylinositol-4-biphosphate, *PKA* protein kinase A, *PLC* phospholipase C, *TATA* TATA box, *Th* gene tyrosine hydroxylase gene)