Ca\(^{2+}\)-dependent Activator Protein for Secretion 1 Is Critical for Constitutive and Regulated Exocytosis but Not for Loading of Transmitters into Dense Core Vesicles\(^*\)

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Although CAPS1 was originally identified as a soluble factor that reconstitutes Ca\(^{2+}\)-dependent secretion from permeabilized neuroendocrine cells, its exact function in intact mammalian cells remains controversial. Here we investigate the role for CAPS1 by generating stable cell lines in which CAPS1 is strongly down-regulated. In these cells, Ca\(^{2+}\)-dependent secretion was strongly reduced not only of catecholamine but also of a transfected neuropeptide. These secretion defects were rescued by infusion of CAPS1-containing brain cytosol or by transfection-mediated expression of CAPS1. Whole cell patch clamp recording revealed significant reductions in slow burst and sustained release components of exocytosis in the knocked cells. Unexpectedly, they also accumulated higher amounts of endogenous and exogenous transmitters, which were attributable to reductions in constitutive secretion. Electron microscopy did not reveal abnormalities in the number or docking of dense core vesicles. Our results indicate that CAPS1 plays critical roles not only in Ca\(^{2+}\)-dependent, regulated exocytosis but also in constitutive exocytosis downstream of vesicle docking. However, they do not support the role for CAPS1 in loading transmitters into dense core vesicles.

Catecholamines such as dopamine and noradrenaline are released from secretory vesicles through regulated and constitutive secretory processes. Distinct types of secretory vesicles account for the storage and secretion of different neurotransmitters and peptides/neuromodulators. Synaptic vesicles are responsible for the storage and secretion of classical neurotransmitters such as glutamate, glycine, and \(\gamma\)-aminobutyric acid, whereas dense core vesicles are responsible for the storage and secretion of peptides and catecholamines. Ca\(^{2+}\)-dependent activator protein for secretion 1 (CAPS1) is a 145-kDa protein purified from the brain and found to be a critical cytosolic factor capable of reconstituting secretion of noradrenaline from dense core vesicles in permeabilized PC12 cells (1–3). Recently, a second CAPS isoform, CAPS2, was identified. CAPS2 is structurally and functionally similar to CAPS1 but exhibits a different cell/tissue and developmental expression (4–6).

CAPS1 and CAPS2 are conserved proteins that have an orthologue in Caenorhabditis elegans (2, 7, 8) and Drosophila (9). In C. elegans, the orthologue of CAPS1 was discovered as unc-31, because the C. elegans with unc-31 gene mutations displays locomotion defects (7, 10). unc-31 mutants were also isolated in the screening of mutants resistant to aldicarb, an ace-ticholinesterase inhibitor, suggesting that Unc-31 is involved in acetylcholine metabolism or secretion (11). In addition, unc-31 mutants appeared to exhibit higher accumulation of serotonin in hermaphrodite-specific motor neurons (12). In Drosophila, depletion of CAPS caused a 50% decrease in glutamatergic transmission at the neuromuscular junctions, but this reduction was not rescued by expression of CAPS in neurons (9). Overall, these results suggest the involvement of CAPS1 in secretory processes. The exact function of CAPS1, however, remains unclear.

The functional analyses of CAPS1 in mammalian secretory cells had been limited. Two studies showed that injection of anti-CAPS antibody inhibit exocytosis in single chromaffin cells (13) and melanotrophs (14), indicating that CAPS1 is required for a late phase of dense core vesicle exocytosis. Other studies demonstrated that recombinant CAPS proteins expressed in COS cells restore secretion from populations of permeabilized PC12 cells (2, 3, 15). A more definitive study was of a CAPS1 knock-out mouse, which was recently published (16). However, homozygous mutant mice died within 30 min after birth, and, therefore, detailed analyses of the roles of CAPS1 in adult neurons and neuroendocrine cells were not feasible. Nonetheless, an initial analysis of the CAPS1-deficient embryonic chromaffin cells unexpectedly showed that CAPS1...
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is primarily involved in loading or maintenance of catecholamines in dense core vesicles, a secretory event upstream from the exocytic steps per se (16). Although these results on the CAPS1 knock-out mice are interesting, they do not provide a satisfactory explanation for the earlier reports of CAPS1 actions in stimulating catecholamine secretion in permeabilized PC12 cells (1, 2, 3, 15). Furthermore, the conclusion from this report is inconsistent with those derived from C. elegans unc-31 mutant studies, which demonstrated defective secretory phenotypes (accumulation of neurotransmitter serotonin in neurons (12) and resistance to aldicarb (11)). This apparent inconsistency in the CAPS1 knock-out mouse study may in part be explained by the fact that, at the embryonic stage of development, CAPS2 is expressed at higher levels than CAPS1 in the adrenal glands (16), and could conceivably exhibit some confounding functional redundancy to CAPS1. In contrast, adult adrenal medullary chromaffin cells do not express CAPS2 at a detectable level (4–6). Indeed, the phenotypes of adult chromaffin cells from CAPS1 heterozygous mouse were apparently different from those of the CAPS1-deficient embryonic cells. They showed reductions in exocytosis and in docking of dense core vesicles but did not exhibit deficits in the loading of catecholamines into vesicles (16). However, the lack of defects in catecholamine loading in the adult heterozygous cells is easily attributable to the presence of residual (50% of the wild-type) CAPS1 in these cells. Thus, it remains unclear whether CAPS1 is critical for loading (or maintenance) of catecholamines into dense core vesicles or for exocytosis from dense core vesicles in intact mammalian cells.

In the current study, we have sought to identify the precise function of CAPS1 in intact mammalian cells by employing the strategy of generating stable PC12 cell lines in which CAPS1 is strongly down-regulated. CAPS2 is not expressed at a detectable level in PC12 cells, as is the case with adult chromaffin cells (4–6). From the analysis of these knockdown cells and the rescue of this loss of CAPS1 function, we strongly suggest that the primary function of CAPS1 is for the secretion of transmitters per se and not for loading of catecholamine into dense core vesicles. We also suggest that CAPS1 is critical not only for regulated exocytosis as previously suggested but also for constitutive exocytosis.

EXPERIMENTAL PROCEDURES

General Materials—We obtained monoclonal antibodies against CAPS1 from BD Bioscience (Mississauga, Ontario, Canada), syntaxin1 (clone HPC-1) from Sigma (Oakville, Ontario, Canada), and SNAP-25 (clone SM1 81) from Covance (Berkeley, CA); polyclonal antibodies against secretogranin II from QED Bioscience (San Diego, CA). Monoclonal antibody (Berkeley, CA); polyclonal antibodies against secretogranin II from Covance (Ontario, Canada), and SNAP-25 (clone SMI 81) from Covance (Ontario, Canada), syntaxin1 (clone HPC-1) from Sigma (Oakville, CA). We used TTCAAGAGA as a linker sequence (18, 19). 64-bp oligonucleotides containing sense and antisense of the target sequences were annealed and subcloned into the BamHI-HindIII sites of pSuper, generating the CAPS1 knockdown plasmid (pSuper-rCAPS1–3). Inserted sequences were verified by sequencing.

Isolation of Stable CAPS1 Knockdown PC12 Cells—Wild-type PC12 cells (which were kind gifts from Dr. Thomas Martin, University of Wisconsin and Dr. Erik Schweitzer, University of California at Los Angeles) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 5% calf serum, 5% horse serum (both from HyClone, Logan, UT), and penicillin/streptomycin (Sigma). To establish the CAPS1 knockdown clone cells, PC12 cells were co-transfected with non-linearized pSuper-rCAPS1–3 (10 μg) and pBabe-puro (1 μg) by electroporation (19). We conducted two independent transfections to isolate knockdown clones and control clones. This is to ensure that the phenotypes observed are independent of screening artifacts. Transfected cells were maintained in growth medium containing 2.5 μg/ml puromycin for more than a month. The growing colonies were picked up with pieces of Whatman paper soaked with Hanks’ buffer containing 1 mM EDTA. Isolated colonies were grown in the growth medium without puromycin in 24-well plates. When the cells became confluent, they were transferred into 2 wells of 6-well plates that contained the growth medium with 2.5 μg/ml puromycin. The cells in one of the 6-well plates were subjected to immunoblot analysis using an anti-CAPS1 monoclonal antibody. Once the down-regulation of CAPS1 was confirmed, the cells in the replica wells were transferred into 10-cm dishes containing growth medium with puromycin. These cells were grown, frozen, and kept in a liquid nitrogen tank until use.

3H]Noradrenaline Release Assays from PC12 Cells—PC12 cells were plated in 24-well plates; 3–4 days after plating, the cells were labeled with 0.5 μCi of [3H]NA (GE Healthcare, Montreal, Quebec, Canada) in the presence of 0.5 mM ascorbic acid for 12–16 h. The labeled PC12 cells were incubated with the fresh complete Dulbecco’s modified Eagle’s medium for 1–5 h to remove unincorporated [3H]NA. The cells were washed once with physiological saline solution (PSS) containing 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl2, 0.5 mM MgCl2, 5.6 mM glucose, and 15 mM HEPES, pH 7.4, and NA secretion was stimulated with 200 μl of PSS, High K+ -PSS (containing 81 mM NaCl and 70 mM KCl), or PSS containing 3 nM of α-latrotoxin (α-LTx, Alomone Laboratories, Jerusalem, Israel). Secretion was terminated after a 15-min (High K+ ) or 10-min (α-LTx) incubation at 37 °C by chilling to 0 °C, and samples were centrifuged at 4 °C for 3 min. Supernatants were removed, and the pellets were solubilized in 0.1% Triton X-100 for liquid scintillation counting.

3H]NA Release Assays Using Permeabilized PC12 Cells—PC12 cells were labeled with 0.5 μCi of [3H]NA in the presence of 0.5 mM ascorbic acid. After washing, the cells were harvested in KGlue buffer (20 mM HEPES, pH 7.1, 120 mM potassium glutamate, 20 mM potassium acetate, and 2 mM EGTA) with 0.1% bovine serum albumin (BSA), mechanically permeabilized (1–3, 15, 20–22), and incubated for 1–3 h on ice in the presence of 10 mM EGTA to extract the cytosolic proteins. Permeabilized PC12 cells were washed three times with KGlue buffer contain-
ing 0.1% BSA. Ca^{2+}-dependent secretion assays were performed in KGlu buffer with 0.07% BSA, 2 mM MgATP, with or without 1 mg/ml brain cytosol, and with or without 1.72 mM CaCl_2 for 20 min at 30 °C. 1.72 mM CaCl_2 in KGlu buffer, which contains 2 mM EGTA, produced ~1–3 μm free Ca^{2+} ([Ca^{2+}]_{free}) (21, 22). Secretion was terminated by chilling to 0 °C, and samples were centrifuged at 4 °C for 3 min. The supernatant were removed, and the pellets were solubilized in 1% Triton X-100 for liquid scintillation counting.

NPY-hPLAP Secretion Assay from PC12 Cells—To measure peptide secretion, we used a plasmid (pCMV-NPY-hPLAP) that enables the expression of neuropeptide-Y fused with a soluble domain of human placental alkaline phosphatase (residues 18–506) (19). CAPS1 knockdown and control cells at 70–80% confluency in 10-cm dishes were transfected with 3 μg of pCMV-hPLAP by electroporation. After 48 h, the cells were harvested and re-plated onto 24-well plates. 6 or 7 days after electroporation, the plated cells were washed once with PSS, and NPY-hPLAP secretion was stimulated with 200 μl of PSS or high K+ (70 mM KCl)-PSS. Secretion was terminated after a 25-min incubation at 37 °C by chilling to 0 °C, and samples were centrifuged at 4 °C for 3 min. Supernatants were removed, and the pellets were solubilized in 200 μl of PSS containing 0.1% Triton X-100. The amounts of NPY-hPLAP secreted into the medium and retained in the cells were measured by the Phospha-Light Reporter Gene Assay System (Applied Biosystems, Foster City, CA). We treated the samples at 65 °C for 30 min to inactivate non-placental alkaline phosphatases and assayed an aliquot (10 μl) for placental alkaline phosphatase activity with the kit. The total volume of the assay was 120 μl. After 5–10 min, chemiluminescence was quantified by using an FB12 luminometer (Berthold Detection Systems, Zylux Corp., Oak Ridge, TN).

Confocal Immunofluorescence Microscopy to Examine the Localization of the Transfected Peptide—Sterilized circular glass coverslips (0.25-mm width and 1.8-cm diameter) were placed in 2.2-cm wells within 12-well cell culture plates. The coverslips were then coated for 1 h with poly-d-lysine (0.1 mg/ml) at room temperature. Both CAPS1 knockdown and control PC12 cells that were electroporated with pVenus-N1-NPY (10 μg) were split onto the coated glass coverslips within each well. Cells were allowed to adhere to the coverslips overnight. The PC12 cells were differentiated on the coverslips for 3–4 days in Dulbecco’s modified Eagle’s medium, which contained 100 ng/ml NGF (Sigma), 1% horse serum, 1% calf serum, and penicillin/streptomycin. The cells were washed with phosphate-buffered saline (PBS), fixed for 15 min with PBS containing 4% paraformaldehyde, and permeabilized with PBS containing 0.2% Triton X-100 and 0.3% BSA for 5 min. Nonspecific sites were blocked for 1 h at room temperature in PBS containing 0.3% BSA. Primary antibodies against secretogranin II (rabbit polyclonal antiserum diluted 1:1000) were diluted in blocking buffer and applied to the permeabilized cells for 1 h at room temperature. Following three washes in blocking buffer, Alexa 568-conjugated goat anti-rabbit antibodies (diluted 1:1000, Invitrogen) were diluted in blocking buffer and applied for 1 h at room temperature. Samples were washed again three times in blocking buffer and mounted in Fluoromount-G reagent (SouthernBiotech, Birmingham, AL). Immunofluorescence staining was recorded with a Zeiss laser confocal scanning microscope (LSM 510) with an oil immersion objective lens (63×).

CAPS1 Expression Construct for the Rescue Experiments—We used mouse CAPS1 cDNA (mKIAA1121, kind gift from Dr. Takahiro Nagase, Kazusa DNA Research Institute, Japan) to generate the expression construct for CAPS1. A 5.4-kb MuniI/BamHI fragment from mKIAA1121 clone was subcloned into EcoRI/BamHI site of pCMV5 generating pCMV-mCAPS1-1. mKIAA1121 encodes a long-splice form of CAPS1. To generate a short-splice form (a more common form) of CAPS1 (2), we conducted PCR and generated pCMV-mCAPS1-2. Mouse cDNA (AGACCGGTTCAGCTTTTTT7) differs from rat cDNA (GGACCGATTCCAGGCTTTC) within the knockdown sequence in three bases (indicated by italics). To further protect the mRNA transcripts transcribed from the mouse CAPS1 expression plasmid from being degraded by the anti-CAPS1 RNA interference machineries already induced within the CAPS1 knockdown cells, we introduced three additional silent nucleotide mutations (SNMs) (AGACCGGTTCAGCTTTTTT7 underlining indicates SNM) within the target sequence in the CAPS1 gene. But the mutation from G to C is to remove the AgeI site but not to increase silent mutations. The resulting mouse CAPS1 sequence in the expression plasmid (pCMV-mCAPS1(SNM)-2) differs from the rat CAPS1 sequence by five bases (all are SNMs) within the knockdown sequence.

Photolysis of Caged Ca^{2+} and C_{m} Measurements—Patch electrodes were pulled from 1.5-mm thin-walled borosilicate glass, coated close to the tip with orthodontic wax (Butler, Guelph, Ontario, Canada), and polished to a tip resistance of 2–4 megohms when filled with intracellular solution. Standard bath solution for the experiments contained (in mM): 138 NaCl, 5.6 KCl, 1.2 MgCl_2, 2.6 CaCl_2, 5 glucose, 5 Heps (pH 7.4 with NaOH). Intracellular solution for flash experiments (in mM): 112 Cs-glutamate, 5 NP-EGTA, 3.7 CaCl_2, 2 Mg-ATP, 0.3 Na_2-GTP, and 0.2 fura-6F (pH 7.2 with CsOH). NP-EGTA and fura-6F were purchased from Molecular Probes (Invitrogen). C_{m} was measured using an EPC-10 patch clamp amplifier (HEKA, Lambrecht, Germany) controlled by the lock-in module of PULSE software. The capacitance traces were imported to IGOR Pro (WaveMetrics, Lake Oswego, OR) for analysis. Flashes of UV light and fluorescence-excitation light were generated as described previously (24). In the flash experiments, exocytosis was elicited by photorelease of caged Ca^{2+} preloaded into the cell via the patch pipette. [Ca^{2+}]_i was measured with the Ca^{2+} indicator dyes fura-2 or fura-6F. [Ca^{2+}]_i was determined from the ratio (R) of the fluorescence signals excited at the two wavelengths (340/380 nm), following the equation (23), [Ca^{2+}]_i = R_{min}/(R_{max} - R_{min}), where R_{max}, R_{min}, and R_{eff} are constants obtained from intracellular calibration as previously described (24).

Measurements of Endogenous Catecholamines from PC12 Cells by HPLC—The measurement of the concentrations of catecholamines was measured using an HPLC system consisting of a delivery pump (Model HP1100, Agilent), a reversed-phase analytical column (Zorbax Eclipse XDB-C8, 150 × 4.6 mm i.d., 5 μm, Agilent), degasser, and a fluorescence detector.
We followed the method developed by Lakshmana and Raju (25), which used an isocratic assay without derivatization. The mobile phase consisted of sodium acetate (0.02 m), methanol (16%), heptane sulfonic acid (0.055%), EDTA (0.2 mm), and dibutylamine (0.01%, v/v). The solution was adjusted to pH 3.92 with α-phosphoric acid. The flow rate was set to 0.9 ml/min. As a standard, noradrenaline, adrenaline, and dopamine were dissolved in 0.1 m PCA at the concentration of 250 ng/ml for each, and the volume of injection was set to 10 μl. The good detection and separation of these compounds were first established at 2.5 ng/10-μl sample volume. We then measured the concentrations of catecholamines in PC12 cells from 10-cm dishes. PC12 cells were harvested in 1 ml of Hank's buffer with 1 mm EDTA, and supplemented with 9 ml of the growth medium. The cells were pelleted by centrifugation, washed once with 1 ml of physiological saline solution, and homogenized in 200 μl of 0.1 m PCA. Insoluble materials were removed by three centrifugations, and 10 μl of the cleared solution was applied to HPLC. We detected the clear peak of noradrenaline (in the range of 2.0 to 40 ng/10-μl sample volume) and dopamine (in the range of 20 to 160 ng/10-μl sample volume). This result confirms that the primary catecholamine in PC12 cells is dopamine and the second most abundant catecholamine is noradrenaline (26). The concentrations of these catecholamines were normalized by the total protein concentrations (in the range of 10 to 30 μg/10-μl sample volume) of each sample of PC12 cells. The total protein concentration was measured by using the Bradford method with bovine serum albumin as the standard.

Measurements of Constitutive Secretion of NPY-hPLAP from PC12 Cells—CAPS1 knockdown and control cells at 70–80% confluency in 10-cm dishes were transfected with 3 μg of pCMV-hPLAP using electroporation. After 48 h, the cells were harvested and re-plated in 300 μl of complete growth medium in 24-well plates. Supernatants were removed, and the cells were solubilized in 300 μl of complete growth medium containing 0.1% Triton X-100 every 24 h. The amounts of NPY-hPLAP secreted into the medium and retained in the cells were measured by the Phospha-Light Reporter Gene Assay System.

Electron Microscopy and Analysis of Dense Core Vesicles—The initial fixation was performed within the 10-cm dishes for 1 h using a 3.2% glutaraldehyde, 2.5% paraformaldehyde fixative mixture in 0.1 m cacodylate buffer (pH adjusted to 7.6). Cells were then pelleted in microcentrifuge tubes and fixed overnight with new fixative. The following day, the pellets were washed and stained with lead citrate for 20 min. Grids were then washed and stained with uranyl acetate for 15 min at room temperature. Grids were then washed and dried once again before loading onto an Hitachi H7000 transmission electron microscope for viewing. Electron micrographs were taken of individual cells within each type of control or CAPS1 knockdown clone. These images were then used for analyzing the docking of dense core vesicles in control or CAPS1 knockdown PC12 cells. Dense core vesicles were identified within the single cell electron micrographs as dark spots of radius between 60 and 120 nm. The distance of each vesicle from the plasma membrane was then calculated for each individual cell. The data from multiple single cell images (n = 8–18) within each control or CAPS1 knockdown subclone were then collated.

RESULTS

Stable CAPS1 Knockdown Cells Exhibit Defects in Catecholamine Secretion—To examine the function of CAPS1, we generated stable PC12 cell lines in which the expression of CAPS1 was strongly down-regulated by an RNA interference strategy. Near total reductions of CAPS1 expression to levels that are almost undetectable using this knockdown strategy was crucial to employ such knockdown models as valid surrogates for the knock-out cell models. We co-transfected PC12 cells with a plasmid expressing a short-hairpin RNA against rat CAPS1 mRNA (pSuper-rCAPS1–3) and a plasmid conferring puromycin resistance (pBabe-puro). Independent colonies of stable transfectants of PC12 cells were isolated after incubation with puromycin for over a month (18, 19). We successfully isolated several independent clones that expressed almost no detectable levels of CAPS1 (Fig. 1A). Control clones were similarly isolated, but an empty pSuper plasmid was used instead of pSuper-rCAPS1–3 for co-transfection with pBabe-puro. At no time did we observe a reduction of CAPS1 expression in the control clones. We also examined the expression of other proteins involved in neurosecretion, including synaptotagmin 1, a major Ca2+ sensor for secretion (27), and neuronal SNARE proteins (syntaxin1, SNAP-25, and synaptobrevin 2) (28). We found no changes in the expression of these proteins (Fig. 1A). Thus, we have generated PC12 cells in which CAPS1 is specifically down-regulated.

We found that the noradrenaline secretory ability of the CAPS1 knockdown cells was significantly diminished in comparison to the control cells. Secretion analyses were performed by labeling the seven pairs of CAPS1 knockdown clones and their paired controls with [3H]NA. Because it is not technically feasible to measure secretion from all the seven pairs of cell lines simultaneously, we paired the knockdown and the control cells and conducted the secretion assay simultaneously for each pair. Simultaneous measurements of secretion from each pair would minimize variations derived from experimental procedures. Fig. 1 (B and C) shows the summaries (means ± S.E.) of the secretion from each pair of the clones in response to stimulation by 70 mm KCl depolarization (n = 9) or 3 nm α-LTx (n = 3–6), respectively. In PC12 cells, α-LTxs can stimulate Ca2+-dependent exocytosis by forming pores on the plasma membrane, which bypass the requirement of Ca2+ to influx via Ca2+-channels that open following depolarization (29). Secretion was consistently decreased in knockdown clones by 45–80% in both high K+ and α-LTxs stimulation experiments when compared with control clones. The results clearly indicate that the defects in high K+-induced secretion are not the result of defects upstream of Ca2+ influx following high K+-induced depolar-
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In the first strategy, we examined the ability of CAPS1-containing brain cytosol to rescue secretion defects in permeabilized knockout cells. If the defects in NA secretion (Fig. 1, B and C) were due to defects in catecholamine loading, it would be unlikely that acute infusion of CAPS1-containing brain cytosol would rescue the secretion defects, because catecholamines were not properly stored in dense core vesicles. However, if the defects in catecholamine secretion in CAPS1 knockout cells were due to defects in secretion processes per se, then the infusion of the brain cytosol would likely restore secretion of CAPS1 knockout cells. We found that these permeabilized CAPS1 knockout cells secreted [3H]NA to the same level as control cells when brain cytosol and Ca2+ were present (Fig. 2). The ability of acute infusion of brain cytosol to completely restore the secretion capability of the CAPS1 knockout cells was strongly indicative that the defects in NA secretion were probably not attributable to defects in the loading of this transmitter into dense core vesicles, but rather appeared to lie in the secretion process itself.

FIGURE 1. Down-regulation of CAPS1 results in defects in catecholamine secretion. A, immunoblot analysis of multiple CAPS1 knockdown cells. 30 μg of total homogenates from the CAPS1 knockdown (KD) and control (C) cells were analyzed by SDS-PAGE and immunoblotting using anti-CAPS1, synaptotagmin 1, syntaxin 1, SNAP-25, and synaptobrevin 2 antibodies. Numbers on the left indicate positions of molecular mass markers. B, NA release was stimulated by 70 mM KCl for 15 min. Seven pairs of knockdown and control clones were examined. The error bars indicate ± S.E. (n = 9). C, NA release was stimulated by 3 nM α-LTx for 10 min. Seven pairs of knockdown and control clones were examined. The error bars indicate ± S.E. (n = 3–6).

FIGURE 2. CAPS1 knockdown cells secrete [3H]NA normally in permeabilized secretion assays. The CAPS1 knockdown cells and the paired control cells were permeabilized and incubated for NA release in the presence and absence of Ca2+ with and without brain cytosol. Six pairs of the clones were examined. Three pairs of the clones are shown on both the top and bottom panels. The error bars indicate ± S.E. (n = 3–6).

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CAPS1 Knockdown Cells Exhibit Reduced Secretion of Exogenously Expressed Neuropeptides—The second strategy for distinguishing the possible role of CAPS1 in catecholamine loading into granules versus a generalized defect in exocytosis per se was to examine if CAPS1 knockdown would influence the
release of exogenously expressed neuropeptide transmitters. Although both neuropeptides and catecholamines are released from the same dense core vesicles (30), they are incorporated into dense core vesicles by totally different mechanisms. If the reduced secretion of catecholamines was primarily due to defects in the loading catecholamines into dense core vesicles, we would not observe defects in secretion of transfected neuropeptides. However, if the reduced secretion of catecholamines reflected a defect in secretion in general, we would see a similar level of decrease in peptide secretion. To facilitate the quantification of the amount of peptide released from the cells as well as that retained in the cells, we transfected the cells with a plasmid that expresses neuropeptide Y-fused with a soluble domain (residues 18–506) of hPLAP (19). By using the quantitative, secretory alkaline phosphatase activity hPLAP. Control cells exhibited robust secretion of transfected NPY-hPLAP in high K+ stimulation (70 mM), reaching 25–35% secretion of the total amount of NPY-hPLAP. However, the CAPS1 knockdown cells secreted much less efficiently, ranging 8–15% secretion of the total NPY-hPLAP (Fig. 3A). Thus, CAPS1 knockdown cells exhibited a strong defect in peptide secretion that is remarkably similar in extent to the deficits in NA secretion (in Fig. 1).

Inhibition of NPY-hPLAP secretion could nonetheless be due to a possible mislocalization of the expressed protein away from the dense core granules. To examine this possibility, NPY was fused with fluorescent protein Venus (17) and expressed in control (C8) and knockdown (KD33) cells. We found that the transfected NPY-Venus showed a strong enrichment at the tip of neurites in both control and CAPS1 knockdown cells. It also exhibited very similar granule-like patterns in the somata of the control and knockdown cells. Furthermore, NPY-Venus proteins were similarly co-localized with secretogranin II, a marker protein for dense core vesicles (Fig. 3B). These results strongly indicate that the transfected NPY-fusion proteins are properly stored in dense core vesicles.

These results taken together indicate that the secretion defects of CAPS1 knockdown cells do not appear to be attributable to defects of loading of catecholamine or peptides into dense core granules, but rather, CAPS1 knockdown cells exhibit a general secretion defect affecting both neuropeptide and catecholamine release from dense core granules.

Reduced Neuropeptide Secretion of CAPS1 Knockdown Is Rescued by CAPS1 Transfection—To examine whether the phenotype of reduced peptide secretion is truly attributable to the down-regulation of CAPS1, we conducted rescue experiments. We examined whether CAPS1 expression in the CAPS1 knockdown cells can restore Ca2+-dependent secretion of NPY-hPLAP. Here, we co-transfected the knockdown cells with the expression construct for mouse CAPS1 (pCMV-mCAPS1(SNM)-2, 50 μg) along with pCMV-NPY-hPLAP (3 μg). To enable CAPS1 expression in the knockdown cells, the expression construct contained SNMs within the CAPS1 sequence of the knockdown plasmids (under “Experimental Procedures”) (19). As a control transfection, the knockdown cells were co-transfected with pcMV-NPY-hPLAP (3 μg) and an empty plasmid pcMV5 (50 μg). As a result of the relatively large size (145 kDa) of the CAPS1 protein, the expression level of CAPS1 resulting from transfection was not very strong, although it was still clearly detectable (Fig. 4A). Nonetheless, co-transfection of CAPS1(SNM) largely restored the Ca2+-dependent release of NPY-hPLAP from the knockdown cells (Fig. 4B). Our results suggest that defects of peptide secretion in CAPS1-knockdown cells are indeed attributable to the down-regulation of CAPS1 protein, rather than the knockdown of unrelated proteins.

CAPS1 Knockdown Cells Exhibit Reduction of Secretory Vesicle Priming and Mobilization to Releasable Pools—Secretion of transmitters from dense core vesicle consists of many steps, including docking and priming of the vesicles, as well as triggering of membrane fusion (exocytosis). We carried out a detailed examination of priming and exocytosis by conducting patch clamp capacitance measurement. Exocytosis was monitored as an increase in the whole cell membrane capacitance (Cm). In response to the step-like [Ca2+] elevations generated by flash photolysis, Cm traces display a rapid, burst-like increase within the first 0.5 s after the flash, followed by a slower sus-

![Image](https://example.com/image.png)
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FIGURE 4. Transfection of CAPS1 restores high K\(^+\)-stimulated exocytosis in the CAPS1 knockdown cells. A, immunoblot analysis of the expression of transfected CAPS1 in the CAPS1 knockdown cells. B, secretion of NPY-hPLAP from the CAPS1 knockdown cells that were transfected with a control vector or pCMV-CAPS1(SNM)-2. The transfected cells were re-plated in 24-well plates and were stimulated for secretion with PSS or 70 mM KCl-PSS for 25 min on the 6th or the 7th days after transfection. Five pairs of knockdown and control clones were examined. The error bars indicate ±S.E. (n = 9).

A

B

FIGURE 5. Marked reduction of exocytosis in CAPS1 knockdown cells. Exocytosis was elicited by flash photolysis of caged Ca\(^{2+}\) and was monitored by whole cell membrane capacitance measurement. A, averaged [(Ca\(^{2+}\)]_i) and capacitance change from control (C46, black line, n = 13) and CAPS1 knockdown (KD77, gray line, n = 15) cells. Arrow indicates the flash time. B, comparison of the exocytotic burst in C46 (black bar) and KD77 (white bar) cells, respectively. The amplitude of the exocytosis burst in KD77 knockdown cells was significantly reduced as compared with that of control cells. Values represent the Mean ± S.E. *, p < 0.05 (Student’s t test). C, the release rate of sustained component in KD77 knockdown cells was also remarkably reduced as compared with that of control cells. Values represent the Mean ± S.E. *, p < 0.05 (Student’s t test).

tained phase of secretion. Secretion typically consists of three components with a triple exponential time course, which we termed the fast burst, the slow burst, and the sustained components, as previously reported (31, 32). The fast burst and slow burst components are generally interpreted as the fusion of docked and primed vesicles from the rapidly and slowly releasable pools, respectively, whereas the sustained components represent refilling of the releasable pools from a large depot pool of vesicles (33). In our experiments, flash photolysis of NP-EGTA was used to induce a step-like, homogenous increase of [Ca\(^{2+}\)], from 200–300 nm to 10–20 µM. The averaged C_m traces were compared between control (C46) and CAPS1 knockdown (KD77) cells responding to similar step-like [Ca\(^{2+}\)] elevations (Fig. 5A). As shown in Fig. 5B, in the control cells, the fast burst component was 271 ± 14 femtofarads (n = 13) with a time constant of ~60 ms, and the slow burst component was 868 ± 65 femtofarads with a time constant of ~300 ms. However, in CAPS1 knockdown (KD77) cells, the fast burst component was 214 ± 9 femtofarads (n = 15) with the time constant of ~100 ms, and the slow burst component was 606 ± 43 femtofarads with the time constant of ~300 ms. Compared with C46 cells, KD77 cells modestly reduced the fast burst amplitude and rate and remarkably reduced the amplitude of the slow burst component (~30%, p < 0.05). Furthermore, we observed a 50% reduction in the release rate of the sustained component. The averaged slope of the sustained component was 43 ± 7 femtofarads/s in C46 cells and 22 ± 3 femtofarads/s in KD77 knockdown cells (Fig. 6C, p < 0.05). These results suggest that CAPS1 is indeed essential for both vesicle priming and refilling of the releasable pool. Although no reductions in capacitance increases were detected in the CAPS1-deficient embryonic chromaffin cells, there were significant reductions in capacitance increases in adult chromaffin cells from CAPS1 heterozygous mice (16). Thus, the phenotypes of our CAPS1 knockdown cells are more similar to those of CAPS1-heterozygous adult cells rather than the phenotypes of CAPS1-deficient embryonic cells. Phenotypic similarities and differences between our CAPS1 knockdown cells and the reported CAPS1-heterozygous adult chromaffin cells will be further discussed under “Discussion.”

Increases in Amounts of Exogenous and Endogenous Catecholamine in CAPS1 Knockdown Cells—In the course of our high K\(^+\) - and α-LTx-stimulated secretion assays (Fig. 1), we found that the counts (DPM) of [3H]NA incorporated into CAPS1 knockdown cells were somewhat higher than control cells. We previously generated several knockdown cells, including RaA knockdown, RaA/RaB-double knockdown cells (19), but have never observed such increases in [3H]NA count or incorporation into the knockdown cells. These increases also do not seem to be artifacts in isolating CAPS1 knockdown clones. We isolated the knockdown cells through two independent transfections and screens; KD4, KD5, KD33, KD77, and KD89 were isolated from one screen and KD204 and
KD232 were isolated from the other. The higher accumulation of [3H]NA is potentially interesting, because in C. elegans, it has been known that unc-31 mutants exhibit somewhat higher accumulation of serotonin in hermaphrodite-specific motor neurons in comparison to the wild type (12). Therefore, we decided to investigate this intriguing phenomenon in more detail. We analyzed the data from high K+ -induced secretion assays of Fig. 1B and displayed the averaged total counts of [3H]NA (i.e. counts of [3H]NA secreted from the cells plus counts of [3H]NA retained in the cells) in each well of 24-well plates for the control and CAPS1 knockdown cells. The total count of [3H]NA per well was indeed significantly higher in the CAPS1 knockdown cells ((5.89 ± 0.49) × 10^4 DPM/well, n = 7) than in the control cells ((3.38 ± 0.56) × 10^4 DPM/well, n = 7) (independent Student’s t test, t_{12} = 2.37, p = 0.036) (Fig. 6A). We originally thought that this higher count of [3H]NA reflected the mislocalization of incorporated [3H]NA. However, as shown in Fig. 6B, we observed that the higher counts of [3H]NA in the knockdown cells also persisted even after mechanical permeabilization of the cells followed by 1–3 h incubation with 10 mM EGTA-containing KGlu buffer and three times washing with KGlu buffer (used in Fig. 2). That is, mechanically permeabilized CAPS1 knockdown cells (7.96 ± 1.49) × 10^4 DPM/assay tube, n = 6) showed higher accumulation of [3H]NA than permeabilized control cells ((3.21 ± 1.00) × 10^4 DPM/assay tube, n = 6). This was also statistically significant (t_{10} = 2.65 and p = 0.024) (Fig. 6B). It is important to note that these higher amounts of [3H]NA in the knockdown cells were secreted normally in permeabilized secretion assays when brain cytosol was present (Fig. 2). Thus, CAPS1 knockdown cells appear to store [3H]NA properly in dense core vesicles but somehow accumulated higher amounts of the transmitter than the control cells.

What is the underlying mechanism for this higher accumulation of [3H]NA in CAPS1 knockdown cells? Although several possibilities exist, we focused on the following two scenarios. In the first scenario, we thought that, as suggested by the CAPS1 knock-out mice study (16), there might be reductions in endogenous catecholamines in dense core vesicles of CAPS1 knockdown cells and that more empty vesicles might allow for better loading of exogenous [3H]NA. In the second scenario, we thought that CAPS1 knockdown cells accumulate more transmitters than control cells due to the reduction in constitutive secretion. To distinguish between these two possibilities, we measured the level of endogenous catecholamines in CAPS1 knockdown and control cells using HPLC with fluorescence detection (25) (Fig. 6, C and D). As previously reported (26), we found that dopamine and NA are the major catecholamines in PC12 cells. The concentrations of dopamine and NA were normalized by the total protein concentrations of each PC12 clones (Fig. 6, C and D). We found that significantly higher amounts of dopamine are present in CAPS1 knockdown cells (4.86 ± 0.64 ng/μg of protein, n = 7) than in the control cells (2.34 ± 0.31 ng/μg of protein, n = 7) (independent Student’s t test, t_{12} = 3.52, p < 0.01) (Fig. 6C). The difference in NA amounts almost reached a significant level (CAPS1 knockdown cells: 0.57 ± 0.14 ng/μg of protein, n = 7; control cell: 0.26 ± 0.04 ng/μg of protein, n = 7, t_{12} = 2.14, p = 0.053) (Fig. 6D). Thus, it is clear that CAPS1 knockdown cells stores higher amounts of total catecholamines (dopamine plus NA) than control cells. Our results suggest that CAPS1 knock-
CAPS1 in Dense Core Vesicle Secretion
down cells retain higher concentrations of endogenous catecholamines, and thus, exclude the first scenario.

Reductions in Constitutive Secretion of Exogenously Expressed Peptides—To explore the second scenario, we sought to measure the constitutive secretion. However, because of the presence of strong catecholamine transporting activities in PC12 cells, it is difficult to measure constitutive secretion of catecholamines. As an alternative method, we measured the constitutive secretion of transfected NPY-hPLAP. In fact, in the NPY-hPLAP secretion assays shown in Fig. 3, we also observed a tendency that the CAPS1 knockdown cells accumulated higher amounts of total NPY-hPLAP than control cells (supplemental Fig. S1). However, the total amounts of NPY-hPLAP were strongly affected by the transfection efficiency for each cell line. As a result, we were not sure whether CAPS1 knockdown cells retained a higher ratio of transfected peptide due to the reduction of constitutive secretion, or they just exhibited a higher transfection efficiency than control cells. To quantify constitutive secretion, we measured the amount of NPY-hPLAP secreted into the medium and the amount retained inside the cells every 24 h, starting 3 days after transfection (i.e. 1 day after re-plating onto 24-well plates, see “Experimental Procedures”) (34). We then calculated the ratio of the NPY-hPLAP retained in the cells over the total NPY-hPLAP. A high ratio would suggest that the cells retain higher NPY-hPLAP due to a reduction in constitutive secretion. For all the pairs of knockdown and control cells, we found that CAPS1 knockdown cells retained higher ratios of transfected NPY-hPLAP inside the cells than control cells (i.e. CAPS1 knockdown cells release lower ratios of transfected NPY-hPLAP). The difference was statistically significant (paired t test, p < 0.05) on the 6th day after transfection for all the pairs (supplemental Fig. S2). When all the data were combined, we clearly observed the higher retention of transfected NPY-hPLAP inside the knockdown cells and this was highly significant (p < 0.01) for the 4th, 5th, 6th, and 7th days after transfection (Fig. 7). These results strongly suggest reduced constitutive secretion in CAPS1 knockdown cells, which explains, at least in part, the higher accumulation of both endogenous and exogenous transmitters in the CAPS1 knockdown cells.

No Morphological Abnormalities in Dense Core Vesicles in the CAPS1 Knockdown Cells—We next explored the possibility that the higher accumulation of catecholamines and transfected peptides might be due to either an increase in number or redistribution of dense core vesicle populations within the cell. On the other hand, our immunoblot analysis showed no increases in the expression of synaptic vesicle proteins (sytotagmin 1 and synaptobrevin 2) (Fig. 1A), which implies that there are no such increases. Nonetheless, we directly examined the morphology of knockdown and control clones through electron microscopy (19) and specifically analyzed whether CAPS1 knockdown would directly influence the number of dense core vesicles and their distribution inside the cell. We found similar numbers of dense core vesicles in the knockdown and control cells, which was ~40–60 vesicles per single-cell electron micrograph (Fig. 8A). Thus, knockdown of CAPS1 did not affect the total number of dense core vesicles. We then quantitatively analyzed the distribution of the dense core vesicles in the knockdown cells and the control cells. In all the clones examined, ~25 to 40% of dense core vesicles were localized within 50 nm from the plasma membrane, and there were no significant differences between knockdown and control clones (Fig. 8B). Thus, we conclude that CAPS1 is not essential for morphological docking of the dense core vesicles. That there were no changes in vesicle docking in CAPS1 knockdown cells also agrees with the results of our rescue experiment in that CAPS1 containing brain cytosol acutely restored secretion from CAPS1 knockdown cells (Fig. 2). Our results strongly suggest that CAPS1 plays a role in exocytosis downstream of vesicle docking.

DISCUSSION
Regardless of accumulating evidence that CAPS1 plays a role in secretion of dense core vesicles, its exact point of action has been controversial. In particular is the finding from CAPS1 knock-out mice, which indicated the primary role for CAPS1 in loading catecholamine into dense core vesicles (16). Because the knock-out mice died just after birth, it was impossible to analyze the function of this protein in adult cells. The lack of defects in exocytosis in embryonic chromaffin cells from CAPS1 knock-out mice is most likely due to the presence of high levels of CAPS2 at this developmental stage. Adult adrenal medullary chromaffin cells do not express CAPS2 at a detectable level, as is the case with PC12 cells (4–6). Thus we explored the possibility that CAPS1 knockdown PC12 cells may serve as a better model in elucidating the exact function of CAPS1 in adult chromaffin cells. As expected, we found strong secretion defects in CAPS1 knockdown cells (Fig. 1). We went on to a rigorous and systematic analysis to determine whether these
secretion defects in knockdown cells were due to defects in loading of catecholamines into the vesicles or defects in the release of transmitters in general from vesicles. Our results indicate that the secretion defects in CAPS1 knockdown cells are attributable to the defects in the release process per se. Firstly, catecholamine secretion defects were completely restored by the acute infusion of CAPS1-containing brain cytosol (Fig. 2). Secondly, the secretion defects were not specific to catecholamines as similar levels of defects were observed for the secretion of transfected peptides as well (Fig. 3A). The transfected peptide was appropriately stored within the dense core vesicles in the knockdown cells (Fig. 3B), and secretion of the peptide was restored through re-expression of CAPS1 into the knockdown cells by transfection (Fig. 4). Thirdly, patch clamp capacitance measurement revealed significant reduction in the slow burst and sustained release components (Fig. 5).

Our CAPS1 knockdown cells do not seem to have problems in loading catecholamines into dense core vesicles. The supporting evidence comes from that catecholamines stored in the knockdown cells underwent normal regulated secretion once brain cytosol and Ca\(^{2+}\) were infused (Fig. 2). On the contrary, the knockdown cells actually retained more endogenous and exogenous catecholamines than control cells (Fig. 6). In contrast, up to 70% of dense core vesicles in CAPS1 knock-out mice at E14 do not contain catecholamines, which led to the conclusion that there was a problem in loading catecholamines into dense core vesicles (16). We do not have a clear idea that explains phenotypic differences between CAPS1-deficient embryonic chromaffin cells and CAPS1-knockdown PC12 cells. One possibility we envision is that the loading deficiency in CAPS1 knock-out mice may be a secondary effect to a developmental problem in these mice. The knock-out mice may suffer from the developmental immaturity of adrenal glands and may not produce enough catecholamines to be loaded into the vesicles during this stage of development. Another possibility is that the requirement of CAPS1 for loading catecholamines into the vesicles may be developmentally regulated. That is, although immature chromaffin cells require the role for CAPS1 in loading catecholamines, but adult cells may dispense CAPS1 for this function.

At which step of secretion does CAPS1 function in intact PC12 cells? Permeabilized PC12 secretion assays showed that secretion processes can be divided into two temporary distinct steps: an ATP-dependent priming step and a Ca\(^{2+}\)-dependent triggering step (20). CAPS1 was identified to be essential in the Ca\(^{2+}\)-dependent step (2, 20). Because then CAPS1 had been considered to function in the triggering step. However, recent and more detailed analysis favors an idea that CAPS1 is critical for the perfusion step, not for the triggering step (3). Our results of patch clamp capacitance experiments performed in CAPS1 knockdown cells showed reductions in the fast and the slow components as well as in the sustained release component, with the slow burst and the sustained release components more significantly affected. These results also support the function of CAPS1 in vesicle priming and refilling of releasable pools in that intact cell. In the case of CAPS1 heterozygous chromaffin cells, the fast and the slow components were reduced by \(\sim 30\%\), but the sustained release component was not affected (16). Thus there is a phenotypic difference in the sustained release component between the CAPS1-knockdown PC12 cells and the CAPS1-heterozygous adult chromaffin cells. The presence...
of the remaining 50% CAPS1 in the heterozygous cells may be sufficient to maintain a normal refilling of releasable pools.

The exocytosis phenotype of our CAPS1 knockdown showed a marked contrast to the phenotype of synaptotagmin I knock-out chromaffin cells. Synaptotagmin I-deficient chromaffin cells showed a selective decrease in the fast burst components, but not in the slower burst or sustained release components (35). The phenotypes of our CAPS1 knockdown cells are more similar to the phenotype of Munc13-1-deficient pancreatic β-cells that show selective reduction in sustained insulin release upon prolonged stimulation (36). Our results suggest a new model in which CAPS1, like Munc13-1, functions in vesicle priming, whereas synaptotagmin 1 functions in the triggering step as a Ca\(^{2+}\) sensor (27).

Unexpectedly, our CAPS1 knockdown cells also revealed an important role for CAPS1 in constitutive secretion. It has been known that mutations in unc-31, an orthologue of CAPS1, in C. elegans showed an increase in serotonin immunoreactivity (12). Our CAPS1 knockdown cells also portrayed a higher accumulation of endogenous and exogenous transmitters. Our results suggest that this higher accumulation of transmitters is due to the reduction in constitutive secretion (Fig. 7). Recent analysis of CAPS2 knock-out mice found that the spontaneous secretion of NT-3 in the culture medium was only ~36% of the level in the wild-type culture medium, but that there was no significant difference between the CAPS2\(^{−/−}\) and wild-type cultures in terms of the total amount of NT-3 in the cell lysates (37). These results suggest the involvement of CAPS2 in constitutive secretion of neurotrophins. Thus, both CAPS1 and CAPS2 are involved in constitutive secretion from dense core vesicles. CAPS1 is a large (145 kDa) protein with multiple domains. An interesting question would be whether the roles for CAPS1 in regulated and constitutive secretions are mediated by the same domains or the distinct domains of this protein. Previous structure/function studies of CAPS1 focused on the Ca\(^{2+}\)-dependent binding of PH domain of CAPS1 to inositol 1,4,5-biphosphate in Ca\(^{2+}\)-dependent-regulated secretion (3, 15), but other domains may mediate the function of CAPS1 in Ca\(^{2+}\)-independent constitutive secretion. The availability of the CAPS1 knockdown PC12 cells will provide a unique opportunity to address these questions. Another interesting question is whether CAPS1 is also involved in the release from small clear vesicles in addition to secretion from dense core vesicles. The unc-31 mutant was also isolated in the screening of mutants resistant to aldicarb (an inhibitor of acetylcholinesterase), which suggests that Unc-31 is involved in acetylcholine pathways (11). In addition, in Drosophila, deletion of CAPS caused a 50% decrease in glutamatergic transmission at the neuromuscular junctions (9). Acetylcholine and glutamate are released from small clear vesicles; this implies that CAPS1 may play roles in secretion not only from dense core vesicles but also from small clear vesicles. PC12 cells are known to contain cholinergic small clear vesicles. Although the measurement of cholinergic secretion from PC12 cells is a challenge, such an analysis would shed new light on the function of CAPS1.

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