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Regulation of Neuropeptide Processing Enzymes by Catecholamines in Endocrine Cells

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ABSTRACT

Treatment of cultured bovine adrenal chromaffin cells with the catecholamine transport blocker reserpine was shown previously to increase enkephalin levels severalfold. To explore the biochemical mechanism of this effect, we examined the effect of reserpine treatment on the activities of three different peptide precursor processing enzymes: carboxypeptidase E (CPE) and the prohormone convertases (PCs) PC1/3 and PC2. Reserpine treatment increased both CPE and PC activity in extracts of cultured chromaffin cells; total protein levels were unaltered for any enzyme. Further analysis showed that the increase in CPE activity was due to an elevated V_{max}, with no change in the K_m for substrate hydrolysis or the levels of CPE mRNA. Reserpine activation of endogenous processing enzymes was also observed in extracts prepared from PC12 cells stably expressing PC1/3 or PC2. In vitro experiments using purified enzymes showed that catecholamines inhibited CPE, PC1/3, and PC2, with dopamine quinone the most potent inhibitor (IC_{50} values of ~50–500 μM); dopamine, norepinephrine, and epinephrine exhibited inhibition in the micromolar range. The inhibition of purified CPE with catecholamines was time-dependent and, for dopamine quinone, dilution-independent, suggesting covalent modification of the protein by the catecholamine. Because the catecholamine concentrations found to be inhibitory to PC1/3, PC2, and CPE are well within the physiological range found in chromaffin granules, we conclude that catecholaminergic transmitter systems have the potential to exert considerable dynamic influence over peptidergic transmitter synthesis by altering the activity of peptide processing enzymes.

Introduction

Peptide hormones and peptide neurotransmitters are typically produced from precursors by the selective action of endo- and exopeptidases. Several peptide processing endopeptidases have been identified, of which prohormone convertase 1/3 [also known as prohormone convertase 1 and 3 (PC1/3)], and prohormone convertase 2 (PC2) are believed to accomplish the majority of peptide precursor cleavage (for review, see Lindberg, 1991; Seidah et al., 1991; Steiner et al., 1992). These enzymes cleave peptide precursors at specific basic residues, generating intermediates with C-terminal basic residues (Cameron et al., 2002). The basic C-terminal residues are subsequently removed by a carboxypeptidase (CP). The major CP involved in neuropeptide biosynthesis is CPE, although a second enzyme, CPD, can also contribute (Fricker, 1988). Both CPE and prohormone convertases have been identified in bovine adrenal chromaffin cells, where they are colocalized within secretory vesicles ("chromaffin granules") along with enkephalins and related peptides (Fricker et al., 1982; Hook and Eiden, 1984; Lindberg, 1986).

The regulation of peptide processing enzymes has been studied under conditions that resemble those of the secretory vesicle. Both PCs and CPE are sensitive to pH, with maximal activities in the acidic range, pH 5 to 6. Whereas all three enzymes are stabilized by the addition of calcium (Nalamachu et al., 1994; Cameron et al., 2002), the presence of calcium is obligatory for PCs for manifestation of enzymatic activity (Steiner, 1998). Other modes of regulation of peptide precursor processing enzymes include binding to membranes, which seems to decrease enzyme activity (Fricker, 1988); proteolytic cleavage (Zhou et al., 1993); feedback inhibition by product (Rhodes et al., 1989); and endogenous inhibitors (Martens et al., 1994; Fricker et al., 2000). However, it is likely that many peptide-processing regulatory mechanisms still remain to be discovered.

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ABBREVIATIONS: PC1/3, prohormone convertases 1 and 3; PC2, prohormone convertase 2; AMC, 7-amino-4-methylcoumarin; CPE, carboxypeptidase E; DA, dopamine; DAQ, dopamine quinone; E-64, *trans*-epoxysuccinic acid; E, epinephrine; GEMSA, guanidinoethylmercaptosuccinic acid; NE, norepinephrine; PC12, pheochromocytoma 12; TBS, Tris-buffered saline; ANOVA, analysis of variance.

Three groups have shown that treatment of adrenal medullary chromaffin cells with reserpine (methyl-11,17α-dimethoxy-18β-[(3,4,5-trimethoxybenzoyl)oxy]-3β,20α-yohimban-16β-carboxylate), an agent that blocks catecholamine entry into granules, increases the production of small enkephalin-containing peptides (Wilson et al., 1980; Eiden and Zamir, 1986; Lindberg, 1986). Reserpine has also been shown to increase the activity of chromaffin cell CPs in a general CP assay (Hook et al., 1985). However, the biochemical mechanisms by which peptide synthesis increases are not clear, and the effects of reserpine on peptide-processing enzymes have not been examined in any cellular system. In the present study, we have tested various mechanisms by which CPE and PC activity could potentially be altered to result in increased levels of final peptide products. The results of these studies suggest that reserpine acts not by increasing PC and CPE biosynthesis but by blocking the profound inhibition exerted by catecholamines on enzyme activity.

Materials and Methods

Cell Culture. Primary cultures of bovine adrenal chromaffin cells were prepared from freshly obtained tissue as described previously (Lindberg, 1986). Cells were cultured on collagen-coated plates: 100-mm plates typically received 3×10^7 cells; 60-mm plates received 10^7 cells, and 12-well plates received 10^6 cells. Three days after plating, cells were treated with either 1 or 10 μM reserpine (Sigma-Aldrich, St. Louis, MO) in growth medium or with control media lacking reserpine. At 24, 48, or 72 h, cells were washed with phosphate-buffered saline and scraped into ice-cold 10 mM sodium acetate buffer, pH 5.5, containing 1 mM phenylmethylsulfonyl fluoride. Cells were frozen before analysis for CPE, PC1/3, and PC2.

PC12 cells stably expressing either mouse PC1/3 or PC2 were generated as described previously (Zhou et al., 1995) and cultured in high-glucose Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 5% horse serum and 10% fetal calf serum. Three days after plating, cells were treated with either 1 or 10 μM reserpine or with control media lacking reserpine. After 48 h, the cells were washed with phosphate-buffered saline and scraped into ice-cold 0.1 M sodium acetate buffer, pH 6 for PC1/3 and pH 5 for PC2, containing 1% Triton X-100, 1 μM *trans*-epoxysuccinic acid (E-64), 1 μM pepstatin, and 1 mM phenylmethylsulfonyl fluoride. For assessment of the effect of L-DOPA (Sigma-Aldrich) on the activity of CPE, PC12 cells were cultured in six-well plates (total $n = 9$ wells). After 3 days, three wells were treated with 5 μM reserpine. Twenty-four hours later, L-DOPA was added to the culture medium (final concentration, 30 μM) of six wells (including the reserpine-treated cells) for a further 24 h. After 48 h total, cells were collected as described above.

Enzyme Assays. For CPE activity determinations, cell extracts were thawed and sonicated for 10 s at 4°C using a probe sonicator. CPE activity was measured using the substrate dansyl-Phe-Ala-Arg, as described previously (Fricker and Snyder, 1983). Typically, 25 μl of cell extract was incubated with 100 μM dansyl-Phe-Ala-Arg in 100 mM sodium acetate, pH 5.5, for 1 h at 37°C. CPE is optimally active at pH 5.5 and is inhibited by 1 μM guanidinoethylmercaptosuccinic acid (GEMSA) (Fricker, 1988). CPE activity in cell extracts was defined as the difference between non-GEMSA/noninhibited enzyme activity and the activity measured in the presence of 1 μM GEMSA. Enzyme activity in experiments using purified CPE was measured in the absence of GEMSA. For the determination of kinetic constants (K_m, V_{max}), substrate concentrations of 200, 100, 50, 25, and 12.5 μM were used. All enzyme determinations were carried out in triplicate, with variations between triplicates typically less than 5%.

The enzyme assays for PC1/3 and PC2 were carried out using 30 μl of clarified adrenal chromaffin detergent cell extract and 15 μl of PC12-PC1 or PC12-PC2 cell extract in a total volume of 50 μl, containing 200 μM fluorogenic substrate, pERTKR-methylcoumarin amide as a substrate and 100 mM sodium acetate, pH 6 (for PC1) and pH 5 (for PC2), with 5 mM $CaCl_2$ and 0.1% Brij 35. Assays were performed in the presence of a protease inhibitor mixture composed of 1 μM pepstatin, 0.28 mM tosylphenylalanyl chloromethyl ketone, 1 μM E-64, and 0.14 mM tosyl lysyl chloromethyl ketone. Released 7-amino-4-methylcoumarin (AMC) was measured with a 96-well plate SpectroMax M2 fluorometer (Molecular Devices, Sunnyvale, CA) using an excitation wavelength of 380 nm and an emission wavelength of 460 nm for 2 h at 37°C. Enzyme activity was measured in triplicate and is given as the mean ± S.D. as a percentage of the control rate (picomoles of AMC released per minute). Specific PC2 activity was assessed by measuring the difference between reactions containing 1 μM 7B2 C-terminal peptide and those lacking the 7B2 C-terminal peptide; this 36-residue peptide represents a specific, nanomolar inhibitor of PC2 (Martens et al., 1994). On the other hand, specific PC1/3 activity is given as the difference between reactions containing 1 μM LLRVKR-amide and those lacking this pro-SAAS-derived peptide, which is a potent and selective inhibitor of PC1/3 (Qian et al., 2000). Specific PC1/3 activity represented 58% of the total activity measured, whereas specific PC2 activity represented 64% of the total activity measured in PC2 reactions.

Enzyme Assays in the Presence of Catecholamines. To investigate whether catecholamines inhibit the activity of proteolytic enzymes, highly purified recombinant 87-kDa PC1/3 (380 nM), PC2 (59 nM), and CPE (1 nM; R&D Systems, Minneapolis, MN) were incubated in the presence and absence of dopamine (DA; Sigma-Aldrich), dopamine quinone (DAQ), norepinephrine (NE; Sigma-Aldrich), and epinephrine (E; Sigma-Aldrich) for the indicated time periods using the assay conditions for each enzyme as described above. The preparation of recombinant mouse PC1/3 and PC2 from Chinese hamster ovary cell-conditioned medium has been described previously (Lamango et al., 1996). Catecholamine stock solutions (50 mM in 0.1 M sodium acetate, pH 5.6) were prepared freshly before each experiment. Dopamine quinone was prepared by incubating 50 mM DA with tyrosinase (500 U; Sigma-Aldrich) for 30 min at room temperature. Epinephrine was used at final concentrations of 1, 5, and 10 mM, whereas norepinephrine and dopamine were used at 1, 2.5, and 5 mM. The final concentrations of dopamine quinone per reaction were 50, 150, and 500 μM. All assays were performed at 37°C in duplicate or triplicate for varying times, as indicated in the respective experiments.

Western Blot Analysis. For the Western blot analysis of CPE, 40 μl of chromaffin cell homogenate or 20 μl of PC12 cell homogenate, respectively, were fractionated on denaturing polyacrylamide gels (10%) using standard procedures. After electrophoresis, proteins were transferred to nitrocellulose and the blots probed with antiserum, at a dilution of 1:1000, raised against the N-terminal region of CPE (Fricker et al., 1990). This antiserum detects both soluble and membrane forms of CPE, as well as precursor forms (pro-CPE). For quantification of CPE in chromaffin cell extract, CPE was detected using ^{125}I-labeled protein A. After washing, the blot was dried and exposed to X-ray film for 1 to 5 days at −90°C with an intensifying screen. The amount of immunoreactive CPE was quantified using an image analysis system. All exposures were in the linear range of the film and camera, as determined using standard curves. For detection of CPE in PC12 cells, blots were incubated with CPE antisera overnight. On the following day, the membranes were washed three times with Tris-buffered saline (TBS) containing 0.05% Tween followed by incubation at room temperature for 1.5 h with horseradish peroxidase-conjugated secondary antibody. The membrane was developed by incubation with SuperSignal West Pico chemiluminescent substrate (Pierce Chemical, Rockford, IL) for 5 min, and the chemiluminescent bands were revealed using HyBlot CL Autoradiography Film (Denville Scientific Inc., Metuchen, NJ).

PC12-PC1/3 and PC12-PC2 cells used for Western blots of PC1/3 and PC2 were scraped with a disposable cell scraper (Greiner Bio-

One, Monroe, NC) in such a way that all cells were detached from the bottom of the dish in 150 μl of ice-cold 1× Laemmli sample buffer. Boiled samples were subjected to electrophoresis on 7.5% Criterion Tris-HCl (Bio-Rad Laboratories, Hercules, CA) SDS gels, followed by Western blotting using antisera against PC1/3 (Vindrola and Lindberg, 1992) and PC2 (Shen et al., 1993). Proteins were transferred from gels to nitrocellulose membranes, and were preincubated in 5% nonfat milk in TBS for 45 min at room temperature before incubation overnight at 4°C with primary anti-PC1/3 or PC2 antisera diluted 1:1000 in milk solution. Thereafter, PC1/3 and PC2 were detected using the horseradish peroxidase conjugate/chemiluminescent substrate system described above.

Monoclonal mouse anti-β-actin antibody (0.5 mg/ml; Sigma-Aldrich) was used as a loading control for normalization of signal. After incubation overnight at 4°C, membranes were washed three times with TBS containing 0.05% Tween followed by incubation at room temperature for 1.5 h using goat anti-rabbit IgG coupled to alkaline phosphatase (1:30,000) in milk. The membrane was washed once with TBS containing 0.05% Tween and twice with TBS alone and then developed with 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt/p-nitro blue tetrazolium chloride.

The density of protein bands was determined using an Alpha Imager 3000 system (Alpha Innotech, San Leandro, CA) and normalized against β-actin. The expression levels of PC1/3 and PC2 were calculated as a normalized ratio between β-actin and PC1/3 or PC2, respectively, and presented either as integrated density of bands (arbitrary units $\times 10^{-5}$) or converted to percentages of nontreated controls.

Isolation and Analysis of CPE mRNA. Total RNA from chromaffin cells was prepared as described previously (Cheley and Anderson, 1984). Cells were extracted with guanidinium isothiocyanate and then mixed with ethanol and stored at −20°C to precipitate the nucleic acids. The precipitate was collected by centrifugation in a microfuge at 4°C, and then dissolved in 200 μl of guanidinium isothiocyanate solution. The samples were extracted with phenol and chloroform, precipitated with ethanol at −20°C, and centrifuged in a microfuge at 4°C. The pellets were washed with 70% ethanol and then resuspended in 20 μl of water. The RNA was quantitated by absorption at 260 nm; the ratio of A_{260} to A_{280} was typically 1.7 for the samples. Equal amounts of RNA (approximately 2 μg) were combined with 10× standard saline citrate buffer and applied to a nitrocellulose filter using a slot blot apparatus. A similar amount of RNA isolated from pituitary was used as a positive control, and RNA isolated from liver was used as a negative control. After the samples were applied, the blots were baked for 2 h at 80°C in a vacuum oven and then probed with CPE riboprobe, as described previously (Fricker et al., 1989).

Statistical Analysis. Data were analyzed with one- or two-way ANOVA followed by the Student-Newman-Keuls multiple comparison test, as appropriate, using a statistical software package (SigmaStat; Systat Software, Inc., San Jose, CA). Where data failed equal variance or normality tests, they were analyzed with one-way ANOVA followed by Dunn's multiple comparison test. A probability value of $P < 0.05$ was considered as statistically significant.

Results

Effect of Reserpine Treatment on CPE, PC1/3, and PC2 Activities in Adrenal Chromaffin Cells. Treatment of chromaffin cells with 1 μM reserpine for 48 or 72 h increased CPE activity by approximately 200% (Fig. 1A). In contrast, treatment for only 24 h did not significantly affect CPE activity. For this experiment, CPE activity was defined as the difference between carboxypeptidase activity measured in the presence of 1 mM Co^{2+}, an activator of CPE, and 1 μM GEMSA, an inhibitor of CPE. A small amount of activity is detected in the presence of 1 μM GEMSA; this activity reflects the lysosomal carboxypeptidase B-like enzyme. Unlike CPE activity, the carboxypeptidase that is active in the presence of GEMSA is not activated by reserpine treatment and even decreases substantially after 24 h of reserpine treatment. The lysosomal enzyme *N*-acetylglucosaminidase was not significantly altered by reserpine treatment (Fig. 1A).

To investigate whether levels of CPE protein were altered by reserpine treatment, extracts were analyzed using Western blots. This analysis showed a single protein with a molecular mass of approximately 52 to 54 kDa (data not shown), consistent with the molecular mass of purified CPE (Fricker et al., 1990). No changes in the levels of CPE protein were detected after 1 μM reserpine treatments of 24, 48, or 72 h (Fig. 1B). This result extends a previous study on reserpine-stimulated carboxypeptidases (Hook et al., 1985) by showing that CPE is the specific carboxypeptidase responsible for this increase. To further examine the mechanism for the reserpine-induced increase in CPE activity, the level of CPE mRNA was determined in control and reserpine-treated cells. No significant change in CPE mRNA was observed after treatment with reserpine for 24, 48, or 72 h. These observations suggest that CPE protein and mRNA levels are not altered by reserpine treatment.

Kinetic analysis of the CPE activity in control reserpine-treated chromaffin cell extracts shows the increase to be due to an apparent V_{max} change and not to a K_m change (Fig. 1C). Treatment of the cells with 1 μM reserpine caused an increase in the apparent V_{max} for CPE from 0.89 to 2.5 nmol/min/10^6 cells when assayed without added $CoCl_2$. The K_m for substrate hydrolysis, 34 to 43 μM, was not substantially altered by reserpine treatment and is comparable with the K_m for dansyl-Phe-Ala-Arg hydrolysis by purified CPE (Fricker et al., 1982).

Because the assay used to detect CPE can also detect CPD, it was necessary to physically separate these two enzymes. Because of its sharp pH optimum at 5.5 to 6.0, CPE elutes from a substrate affinity column at pH 8.0, whereas the related CPD remains bound because of its broader pH optimum (Fricker, 1988). CPE and CPD from reserpine-treated and nontreated chromaffin cells were separated using benzoyl-arginine Sepharose affinity columns and subjected to enzymatic assays. Whereas reserpine treatment increased the activity of CPE in chromaffin cell extracts, there was no effect on the enzymatic activity of the related enzyme CPD (Fig. 1D). Thus, reserpine seems to specifically affect the vesicular carboxypeptidase CPE.

In addition to the pronounced effect of reserpine on the enzymatic activity of chromaffin cell CPE, reserpine treatment also affected the activity of PCs 1/3 and 2. The enzymatic activity of PC1/3 was slightly increased when cells were incubated for 48 h with 1 μM reserpine; however, PC1/3 activity was increased by 6-fold when cells were treated with 10 μM reserpine (Fig. 1E). The same trend was observed for the activity of PC2 (Fig. 1F), in which a 48-h treatment of chromaffin cells with 1 μM reserpine led to an increase in activity of approximately 40%. When cells were incubated with 10 μM reserpine, a 3.5-fold increase in PC2 activity was observed.

Effect of Reserpine Treatment of PC12 Cells on Endogenous CPE and PCs. The observed effect of reserpine treatment on CPE, PC1/3, and PC2 enzymatic activity in primary bovine adrenal chromaffin cells could be reproduced by reserpine treatment of rat

adrenal pheochromocytoma cells (PC12) stably transfected with either PC1/3 (Fig. 2, left) or PC2 (Fig. 2, right). Twenty-four hours of incubation with 5 μM reserpine led to an approximately 2-fold increase in enzymatic activity of endogenously expressed CPE in PC12-PC1/3 (Fig. 2A) and PC2 (Fig. 2B) cells. Again, this effect was not accompanied by an increase in CPE protein expression, as demonstrated by Western blot analysis; levels of CPE normalized against β-actin were nearly unchanged (93%, PC12-PC1/3 cells; and 97%, PC12-PC2 cells) in comparison with untreated controls (Fig. 2, A and B, bottom). In a second set of experiments, prohormone convertase activity was measured after a 24-h treatment of PC12 cells with reserpine. In both PC12-PC1/3 and PC12-PC2 cells, exposure to either 1 to 10 μM reserpine caused an increase in the enzymatic activity of the respective transfected convertase (Fig. 2, C and D). Similar to CPE protein levels, the expression of PC1/3 and PC2 protein was relatively unaffected by treatment with reserpine (Fig. 2, C and D, bottom). Western blot analyses showed only a small increase in PC1/3 expression (20–22% increase) and no significant changes in PC2 expression.

Catecholamines Affect Purified CPE, PC1/3, and PC2 Activity In Vitro. Preincubation of recombinant CPE, PC1/3 and PC2 with catecholamines in vitro inhibits the proteolytic activities of all enzymes. A 2-h preincubation of CPE with increasing amounts of DA (1–5 mM) or DAQ (50–500 μM), or a 1-h preincubation with either

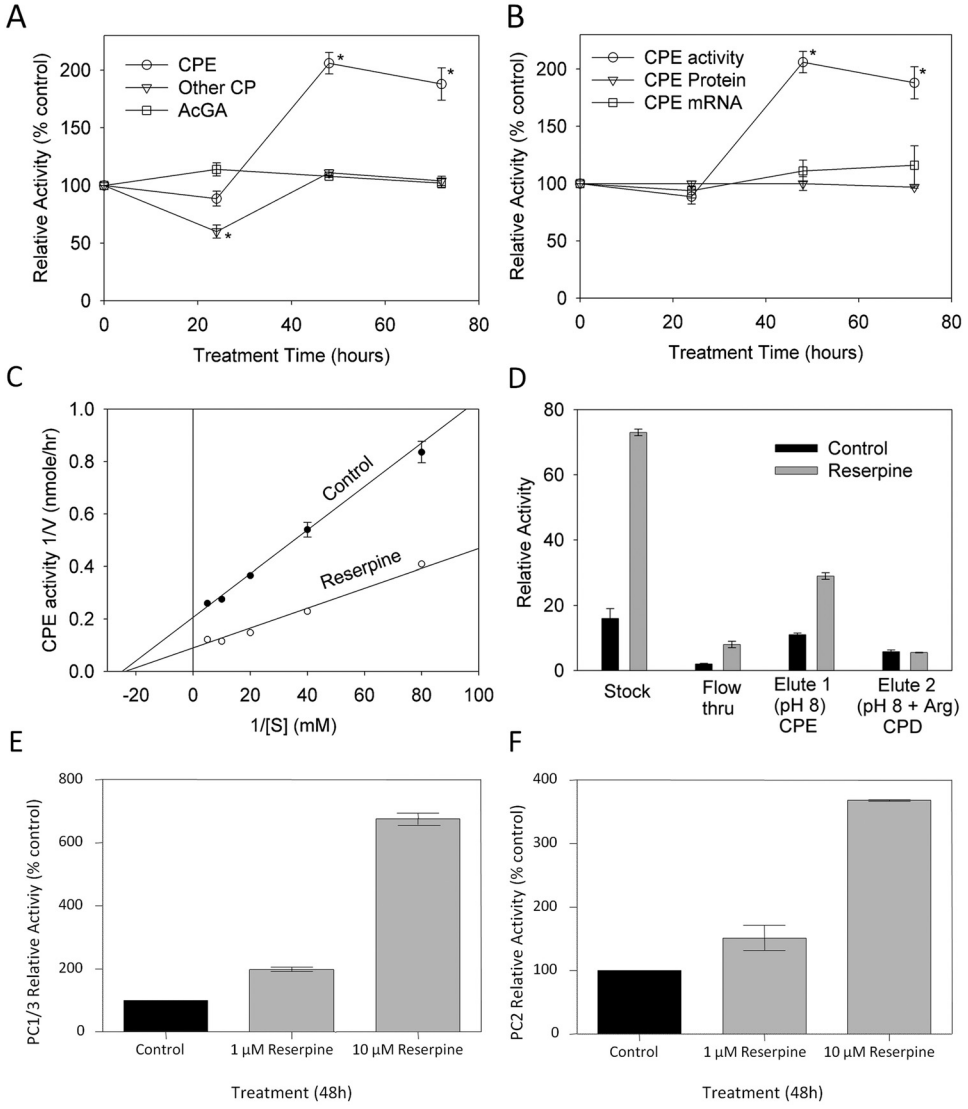

Fig. 1. Effect of reserpine treatment on CPE, PC1/3, and PC2 activities in adrenal chromaffin cells. A, Reserpine treatment increases the enzymatic activity of CPE after 72 h but does not affect the activity of another carboxypeptidase (CP), or of *N*-acetylglucosaminidase. For CPE and other CPs, $n = 18$ for the controls, $n = 6$ for 24-h, $n = 3$ for 48-h, and $n = 9$ for 72-h groups. For *N*-acetylglucosaminidase, $n = 6$ for control, and $n = 3$ for treated groups. $*$, statistically different from control ($P < 0.01$) using Student's t test. Error bars show S.E.M. B, lack of effect of reserpine treatment on levels of CPE immunoreactivity and mRNA. CPE protein levels were assessed by Western blotting, and CPE mRNA was determined using slot blots, as described in *Materials and Methods*. Error bars show S.E.M.; $n = 3$ for all groups. C, the velocity of the CPE reaction increases in the presence of reserpine (Lineweaver-Burk analysis). V is expressed in nanomoles of product per hour per 2×10^4 cells. Error bars show the S.D. ($n = 6$). CPE activity is calculated from the difference between carboxypeptidase activity measured in the presence or absence of GEMSA (without added $CoCl_2$). D, reserpine treatment affects CPE activity and not the related carboxypeptidase D. Control and reserpine-treated chromaffin cell extracts were purified on a benzoyl-arginine Sepharose affinity column, which can be used to physically separate CPE and CPD, allowing for their selective measurement. CPE activity elutes from the column at pH 8.0 (elute 1) and shows a difference between reserpine-treated and nontreated control extracts. CPD remains bound under this condition but can be eluted with a combination of pH 8.0 and arginine. Note the similar levels of CPD activity between the control and reserpine-treated extracts. Error bars show S.E.M. for triplicate determinations. E and F, reserpine increases the enzymatic activity of PC1/3 and PC2 present in chromaffin cell extracts. Cells were incubated with reserpine or vehicle for 2 days. Results are presented as the mean ± S.E.M., as the percentage of control rate (PC1/3 control rate = 22 pmol AMC/min; PC2 control rate = 24 pmol/min; n = 3 per group).

NE (1–10 mM) or E (1–5 mM) resulted in a significant and dose-dependent decrease in CPE activity (Fig. 3, A–D; $P = 0.001$ at highest concentrations). This effect was also time-dependent, as shown in Fig. 3E; here, CPE was preincubated with relatively low amounts of DA (1 mM), DAQ (100 μM), NE (1 mM), or E (1 mM) for time periods up to 12 h before assay for CPE activity. Apart from DAQ, of the three catecholamines tested at 1 mM, NE effects were the most pronounced, resulting in >50% inhibition within 1 h. DAQ inhibition was also prominent, especially considering the 10-fold lower concentration of this compound relative to the other catecholamines in this experiment. All catecholamines completely inhibited CPE activity after the 12-h preincubation (Fig. 3E).

Inhibition of enzymatic activity was observed when purified PC1/3 (Fig. 4, left) and PC2 (Fig. 4, right) were preincubated with increasing concentrations of catecholamines. Dopamine significantly inhibited PC1/3 (Fig. 4A; $P = 0.001$ at 5 mM) and PC2 (Fig. 4B; $P = 0.001$ at 5 mM). Dopamine quinone inhibited PC1/3 activity (Fig. 4C; $P = 0.001$ at 500 μM) and at the same time very profoundly inhibited PC2 activity in a dose-dependent manner (Fig. 4D; $P = 0.001$ at 500 μM). Preincubation of PC1/3 and PC2 with either NE or E inhibited the activity of both enzymes in a dose-dependent manner (Fig. 4, E–H; $P = 0.001$ at highest concentrations). However, at comparable catecholamine concentrations, the overall inhibition of PC2 was more pronounced than the overall inhibition of PC1/3, suggesting greater sensitivity of PC2 to catecholamines.

Effect of L-DOPA Treatment of PC12 Cells on Endogenous CPE. To provide further evidence linking cellular catecholamine concentrations to CPE activity levels, CPE activity was measured in extracts prepared from PC12 cells treated with 30 μM L-DOPA for 24 h (to increase cellular catecholamine levels). The specific CPE activity decreased by ~25% compared with untreated controls (Fig. 5, control versus L-DOPA, $P = 0.042$, ANOVA). Non-GEMSA-inhibited and nonspecific activity, presumably due to lysosomal carboxypeptidases, did not change (data not shown). The usual increase in CPE activity was observed when cells were preincubated with 5 μM reserpine before the addition of L-DOPA to the medium (L-DOPA with versus without reserpine, $P = 0.001$, ANOVA), indicating that reserpine effectively blocked entry of the L-DOPA into the CPE-containing compartment. This experiment provides support for the notion that catecholamine levels, rather than a nonspecific effect of reserpine, are responsible for the decreased enzyme activity observed in our study.

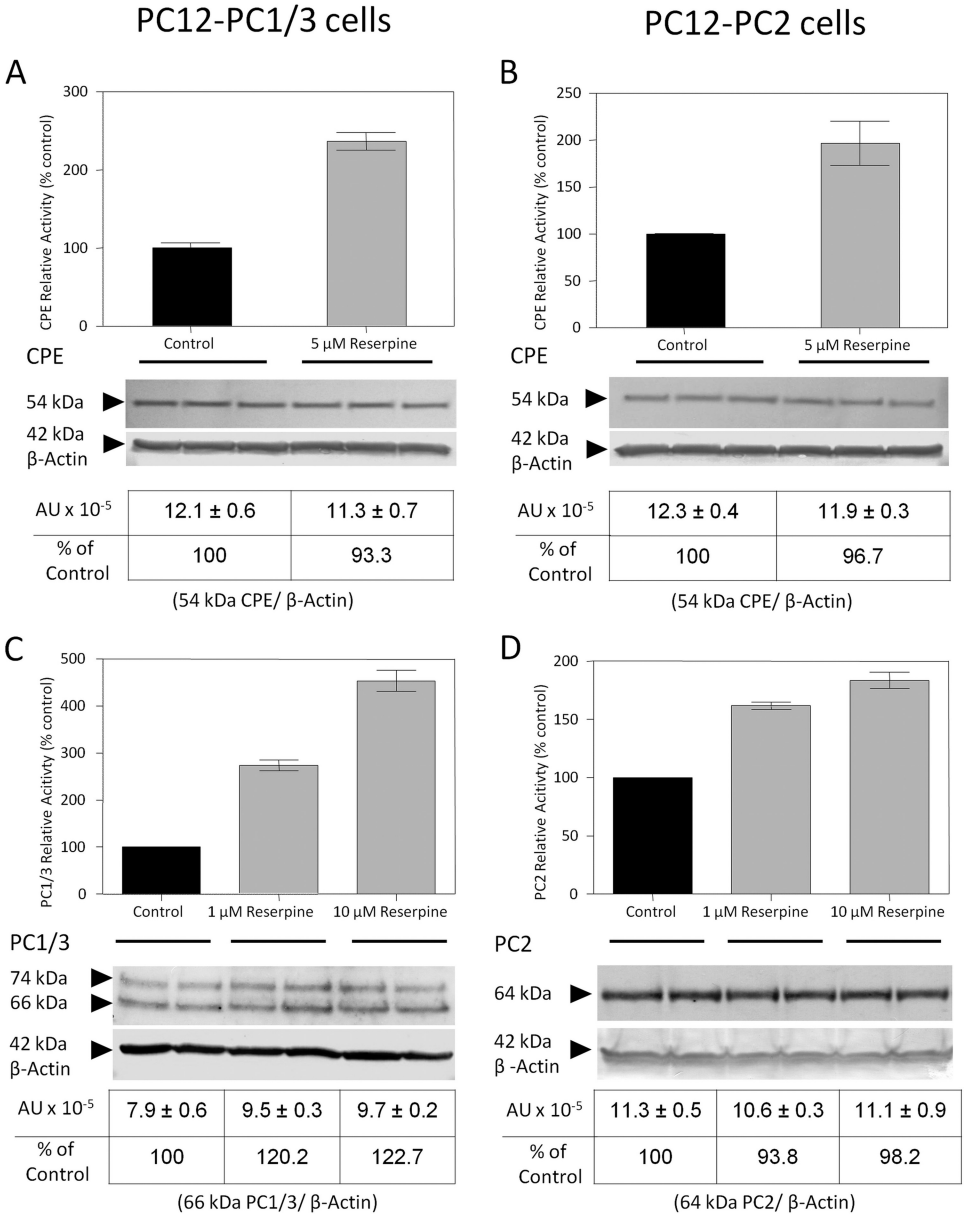

Fig. 2. Reserpine treatment of adrenal PC12 cells increases endogenous CPE and PC activity but has no effect on protein expression. A to D, bar charts showing enzyme activity of CPE, PC1/3, and PC2 in PC12 cells treated with reserpine or vehicle; quantification of enzyme protein levels is presented in the lower portion of each figure. Quantitative analysis of enzyme protein is presented after normalization of enzyme immunoreactivity for β-actin immunoreactivity within the same samples and is expressed as arbitrary units (AU) and as a percentage of nontreated controls. Values represent the mean ± S.E.M. as a percentage of the control rate. Left, PC1/3-overexpressing PC12 cells treated with reserpine for 48h. A, CPE activity increases in the presence of 5 μM reserpine ($n = 3$ per group), whereas CPE protein expression remains unchanged. C, PC1/3 activity increases after treatment of cells with 1 μM and 10 μM reserpine, whereas protein levels are only slightly increased (control rate = 32 pmol AMC/min). Right, PC2-expressing PC12 cells treated with reserpine for 48 h. B, CPE activity increases in PC12-PC2 cells in the presence of 5 μM reserpine ($n = 3$/group). CPE protein levels remain unchanged. D, PC2 activity is increased after treatment with either 1 or 10 μM reserpine; PC2 protein levels are unaltered (control rate = 54 pmol AMC/min).

Mechanism of Inhibition of CPE by Catecholamines. To test whether the observed inhibition is reversible or irreversible, recombinant CPE (Fig. 6) was preincubated for 2 h with selected catecholamines followed by sequential dilution (1:2, 1:10, and 1:100) of the enzymatic reactions before assay. Dopamine (5 mM; $P = 0.001$), DAQ (50 μM; $P = 0.001$), NE (5 mM; $P = 0.001$), and E (5 mM; $P = 0.001$) significantly inhibited CPE activity when undiluted. With increasing dilution, the specific activity of CPE preincubated with DA, NE, and E regained values comparable with those of nontreated CPE (see 1:100; DA, $P = 0.872$; NE, $P = 0.906$; E, $P = 0.452$), suggesting reversibility of inhibition. However, DAQ-treated CPE remained inhibited (DAQ, $P = 0.004$) independent of dilution, suggesting an irreversible inhibition mechanism. It is notable that CPE displays increased basal activity when diluted, an effect that has been observed in other experiments (L. D. Fricker, unpublished data).

In general, similar results were observed when catecholamines were incubated with PC1/3 or PC2 and then diluted, with DAQ-incubated enzymes showing an irreversible loss of enzyme activity and the other catecholamines generally showing reversible inhibition (data not shown). In contrast to CPE, however, the activities of both endopeptidases were highly variable when measured after dilution. This effect may be attributable to their relative lability compared with CPE (i.e., the greater sensitivity of PCs to the extended incubation times required for the dilution paradigm). In a set of seven dilution experiments, PC1/3 inhibition by DA, NE, and E was restored in >85% of the samples by dilution, returning to levels of nontreated PC1/3, whereas DAQ-induced irreversible inhibition of PC1/3 was observed in approximately 60%. DAQ-incubated PC2 remained significantly inhibited in two thirds of the experiments at a 1:20 dilution. However, the activity levels of DA-, NE-, and E-exposed enzymes varied after dilution and no consistent trend regarding irreversibility was observed (i.e., 50% remained inhibited). It is possible that the irreversible inhibition of PC1/3 and PC2 by catecholamines other than DAQ that we observed in some cases is also ultimately due to quinone formation, because norepinephrine and epinephrine can also form quinones (Manini et al., 2007).

Discussion

It has been shown previously that enkephalin levels increase severalfold when adrenal chromaffin cells are treated with catecholamine transport blockers such as reserpine (Wilson et al., 1980; Eiden et al., 1984; Lindberg, 1986). The reason for this dramatic increase in peptide production, however, has remained unclear. In the present study, we have investigated the regulatory effect of reserpine on the activity of the three enzymes, PC1/3, PC2, and CPE, which are responsible for generating mature enkephalins from proenkephalin. We compared the direct effects of various catecholamines on the activity of these enzymes in vitro to the effects of reserpine on these enzymes in two cell culture systems.

Two possible explanations for reserpine-increased peptide

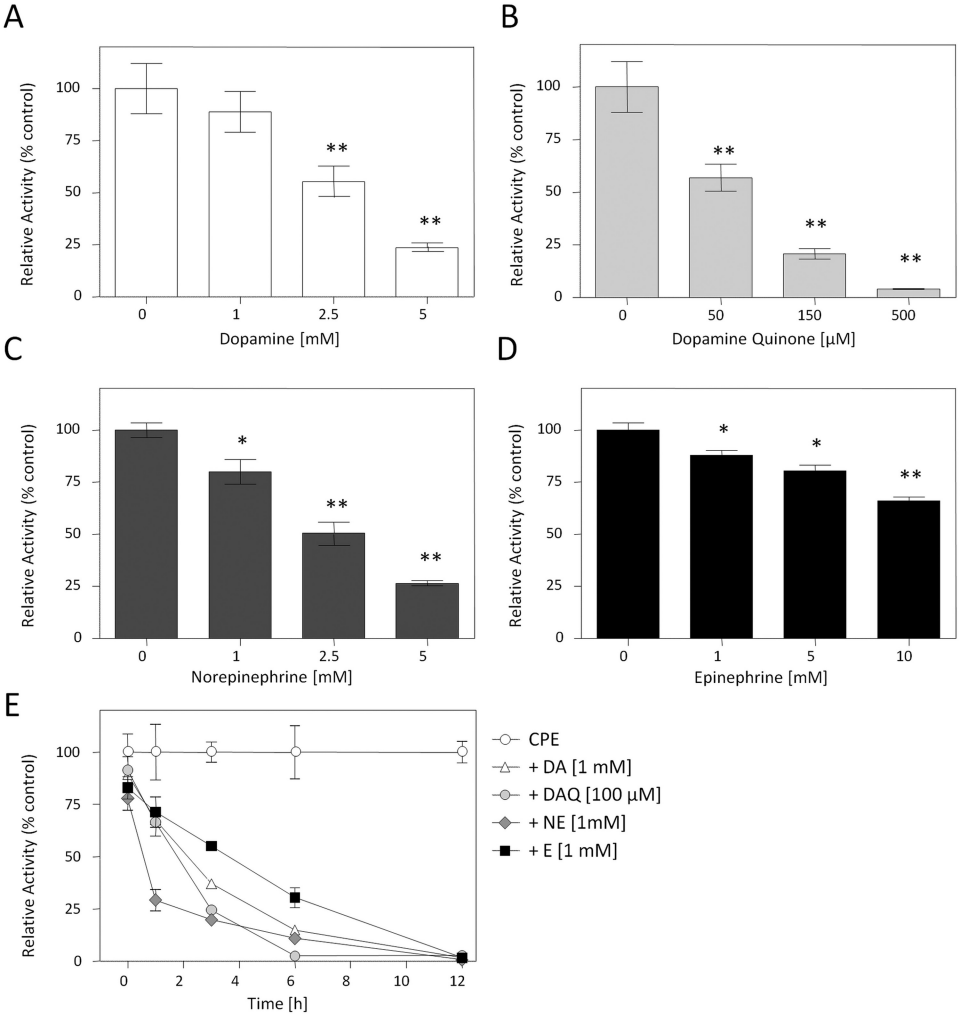

Fig. 3. Catecholamines affect purified CPE activity. Reactions were preincubated with different concentrations of catecholamines for 2 h. Increasing concentrations of catecholamines result in decreased CPE activity ($n = 6$/group). A, open bars, dopamine. B, light gray bars, dopamine quinone. C, dark gray bars, norepinephrine; D, black bars, epinephrine. E, time-dependence of the catecholaminergic inhibition of CPE over 12 h. Whereas dopamine quinone in relatively low concentrations lead to a near complete inhibition of CPE activity after 6 h, dopamine, norepinephrine, and epinephrine inhibited enzyme activity after 12 h. Results are presented as the mean ± S.D., as a percentage of control rate ($n = 3$ per time point). **, $P < 0.01$ and *, $P < 0.05$; statistically different from CPE activity in the nontreated group using ANOVA.

production include changes in the affinity of processing enzymes and/or the synthesis of increased amounts of processing enzymes. A prior report indicated that reserpine treatment increases CP activity in chromaffin cells by lowering its K_m (Hook et al., 1985); however, these conclusions were based on an assay that can also detect CPD and lysosomal CPs. By contrast, we found no reserpine-induced effects on the CPE K_m, but a greatly increased V_{max}. In agreement with Hook et al. (1985), we found that reserpine stimulation of CPE activity in chromaffin cells is not accompanied by increased CPE protein; our mRNA analysis supports this finding. The observation that reserpine does not increase CPE protein levels is further supported by a report showing that GEMSA binding in the rat adrenal is not changed after reserpine treatment (Strittmatter et al., 1985). Analogously to CPE, we found that the activity of cellular PC1/3 and PC2 expressed in both bovine adrenal chromaffin cells, and in convertase-transfected PC12 cells, also increases dramati-

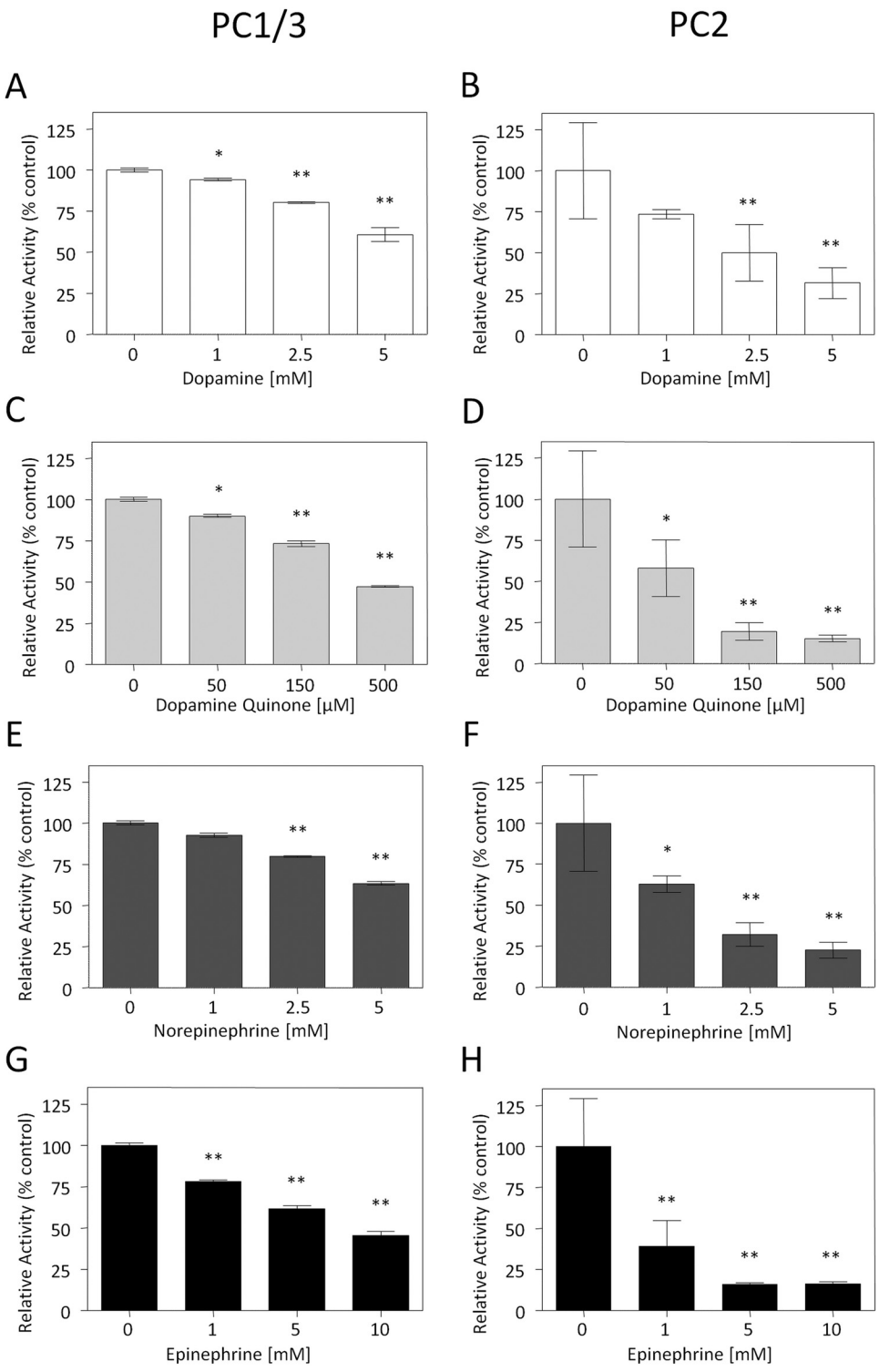

Fig. 4. Catecholamines decrease the activity of purified PC1/3 (left) and PC2 (right). A to D, increasing concentrations of dopamine and dopamine quinone decrease PC1/3 and PC2 enzymatic activity in reactions incubated for 2 h. E to H, norepinephrine and epinephrine significantly inhibit enzyme activity of both convertases. Values are presented as means ± S.D., percentage of controls (n = 3/group). **, $P < 0.01$ and *, $P < 0.05$; statistically different from PC1/3 (control rate = 3 pmol AMC/min) and PC2 (control rate = 8.6 pmol AMC/min) activity in the nontreated control group using ANOVA.

cally after reserpine treatment without any alterations in protein expression. Because all three enzymes are found within secretory granules, we suggest that the reserpine-induced increase in CPE, PC1/3, and PC2 activity is granule-specific, rather than representing a nonspecific increase in cellular enzyme activity. This idea is supported by the finding that three other enzymatic activities, lysosomal carboxypeptidase B-like enzyme, *N*-acetylglucosaminidase, and CPD are not elevated by reserpine treatment.

The time course of reserpine activation yields further clues as to its mechanism of action on peptide-processing enzymes. Our finding that the enzymatic activities of CPE, PC1/3, and PC2 are not influenced in the initial 24 h of reserpine treatment, which would presumably contain similar levels of the drug as the 48 and 72 h samples, is consistent with our prior time course studies on reserpine-induced changes in peptide production (Lindberg, 1986) and suggests that reserpine itself does not directly increase granular enzyme activity. Because reserpine blocks the vesicular monoamine transporter, which leads to a depletion of granular monoamine stores (Kirshner et al., 1963), the observed timing of enzyme activation may reflect an indirect effect on processing enzymes caused by a gradual reduction in intragranular catecholamine levels. In support of this idea, Wolkersdorfer et al. (1996) found that the activity of partially purified PC1/3 decreased when this enzyme was incubated with norepinephrine or epinephrine in vitro. Our present in vitro data showing that catecholamine concentrations as low as 1 mM can directly inhibit not only highly purified PC1/3 but also PC2 and CPE provide substantial evidence that the loss of inhibitory catecholamines represents the primary underlying reason for reserpine-mediated increases in processing enzyme activity. This notion is further supported by the finding that exposure of PC12 cells to a dopamine precursor resulted in lowered CPE activity. Our work also extends these in vitro results to other catecholamines such as dopamine and dopamine quinone and shows that all of these catecholamines directly inhibit not only PC1/3 but also CPE and PC2 in a time-dependent manner in vitro. Physiological catecholamine concentrations in chromaffin granules are quite high, approximately 0.6 M (Winkler, 1976), suggesting that tonic inhibition of peptide processing enzymes by catecholamines may represent a physiologically relevant phenomenon. Whereas structure-function studies remain to be performed, we note that serotonin did not inhibit PCs at a concentration of 5 mM (results not shown) and L-DOPA was not inhibitory, supporting distinct structural requirements for inhibition.

An important question remains as to the molecular mechanism by which catecholamines can inhibit the activity of such diverse enzymes as CPE, PC1/3, and PC2; CPE is a metallopeptidase, whereas PC1/3 and PC2 are serine proteases. Catecholamine-induced increases in CPE activity cannot be due to altered processing of pro-CPE into CPE, because both of these forms of CPE have comparable activities (Parkinson, 1990) and because the effect occurs in vitro with purified mature CPE. The fact that catecholamine inhibition occurs in vitro with purified enzymes also argues

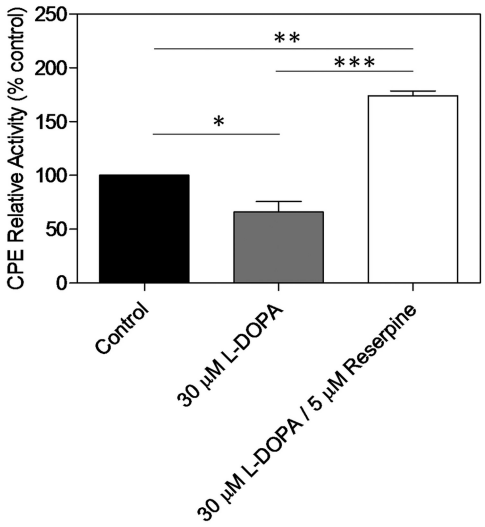

Fig. 5. L-DOPA treatment of PC12 cells decreases endogenous CPE activity. CPE activity in PC12 cells decreases after 24 h in the presence of 30 μM L-DOPA in the medium (n = 3/group). CPE activity increases when PC12 cells are preincubated for 24 h with 5 μM reserpine before the addition of L-DOPA for another 24 h (n = 3/group). ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$; statistically different from control (control rate = 29 pmol AMC/min) using ANOVA.

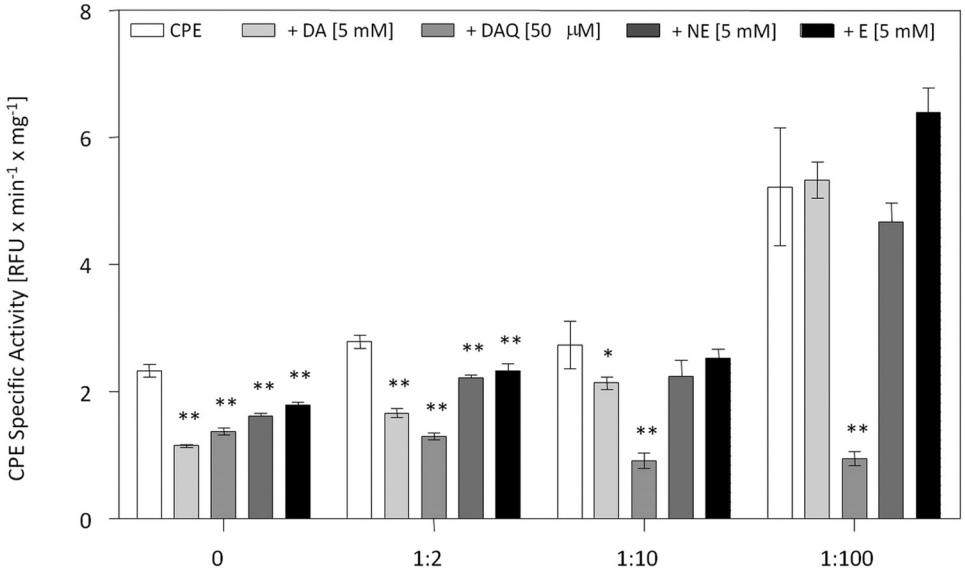

Fig. 6. Catecholaminergic inhibition of CPE by dopamine, norepinephrine, and epinephrine is reversible, whereas inhibition by dopamine quinone is irreversible. CPE was incubated for 6 h at 37°C; reactions were then sequentially diluted and the specific activity [relative fluorescence units per minute per milligram] of untreated CPE was compared with diluted catecholamine-treated enzyme (n = 3/group). Dopamine quinone inhibits CPE activity regardless of the dilution factor. Whereas treatment of CPE with dopamine, norepinephrine, and epinephrine decreases enzymatic activity initially, enzyme activity can be restored by dilution to levels comparable with those of untreated CPE. Results are presented as mean ± S.D. **, $P < 0.01$, and *, $P < 0.05$; statistically different from enzyme activity in the nontreated group using ANOVA.

against the potential contribution of cellular post-translational modifying enzymes, such as kinases, as regulatory mechanisms in reserpine effects; this is in agreement with the finding that the apparent molecular weights of CPE in control and reserpine-treated samples are similar. We propose, based on our observation that the inhibition of CPE by dopamine quinone is dilution-independent, that this compound may form a covalent bond with CPE. This observation is in line with reports demonstrating that dopamine quinones irreversibly alter protein function through formation of protein-5-cysteinyl-catechols, thus permanently impairing enzyme activity (Asanuma et al., 2003; Van Laar et al., 2009). Based on the time- and concentration-dependence of inhibition, we surmise that other catecholamines may also form time-dependent noncovalent allosteric or competitive complexes that inhibit all three enzymes. Considering the fact that PC1/3, PC2, and CPE bind only substrates and inhibitors containing positively charged amino acids, we disfavor the possibility of a direct interaction between catecholamines and the enzyme active sites (which would lead to a competitive type of inhibition). We suggest instead that an allosteric interaction is more likely. Catecholamines induce significant conformational changes in fibrinogen (Martini et al., 2007), and Li et al. (2004) reported that catecholamines and especially oxidized products such as dopamine quinone can disaggregate Aβ peptide fibrils. We propose that the potential mechanism of catecholamine inhibition involves binding of one or more catecholamine molecules to allosteric sites, leading to conformational changes affecting enzyme activities.

We further suggest that the inhibition of peptide-processing enzymes by catecholamines is part of a self-regulating mechanism that balances production and storage of catecholamines and neuropeptides within the same vesicles of the secretory pathway. This idea is supported by our and others' previous findings that reserpine-induced reduction of intravesicular catecholamine levels is associated with increased synthesis and release of processed neuropeptides (Eiden and Zamir, 1986; Lindberg, 1986; Wilson, 1987; Laslop et al., 1994). In turn, neuropeptides are known to regulate the synthesis and release of cosecreted catecholamines. For example, it has been reported that vasoactive intestinal peptide, secretin, and neuropeptide Y increase catecholamine synthesis by activation of tyrosine hydroxylase in human adrenal chromaffin cells (Ip et al., 1984; Cavadas et al., 2001; Rosmaninho-Salgado et al., 2009). The regulatory circuit is completed when this neuropeptide-triggered increase in catecholamines eventually leads to a reduction in neuropeptide production, accomplished by catecholaminergic inhibition of neuropeptide processing enzymes, as demonstrated in the present study. Whether the strong catecholaminergic inhibition of neuropeptide processing enzymes we observe in adrenal chromaffin and PC12 cells can be extended to catecholaminergic and peptidergic neurons of the central nervous system represents an interesting topic for further study.

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Authorship Contributions

Participated in research design: Fricker, Helwig, Lindberg, and Vivoli.

Conducted experiments: Fricker, Helwig, Lindberg, and Vivoli.

Performed data analysis: Fricker, Helwig, and Vivoli.

Wrote or contributed to the writing of the manuscript: Fricker, Helwig, Lindberg, and Vivoli.

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