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Internal cleavage of the inhibitory 7B2 carboxyl-terminal peptide by PC2: A potential mechanism for its inactivation

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ABSTRACT **The neuroendocrine protein 7B2 contains two domains, a 21-kDa protein required for prohormone convertase 2 (PC2) maturation and a carboxyl-terminal (CT) peptide that inhibits PC2 at nanomolar concentrations. To determine how the inhibition of PC2 is terminated, we studied the metabolic fate of the 7B2 CT peptide in RinPE-7B2, AtT-20/PC2–7B2, and αTC1–6 cells. Extracts obtained from cells labeled for 6 h with [^{3}H]valine were subjected to immunoprecipitation using an antibody raised against the extreme carboxyl terminus of r7B2, and immunoprecipitated peptides were separated by gel filtration. All three cell lines yielded two distinct peaks at about 3.5 kDa and 1.5 kDa, corresponding to the CT peptide and a smaller fragment consistent with cleavage at an interior Lys-Lys site. These results were corroborated using a newly developed RIA against the carboxyl terminus of the CT peptide which showed that the intact CT peptide represented only about half of the stored CT peptide immunoreactivity, with the remainder present as the 1.5-kDa peptide. Both peptides could be released upon phorbol 12-myristate 13-acetate stimulation. We investigated the possibility that PC2 itself could be responsible for this cleavage by performing *in vitro* experiments. When ^{125}I-labeled CT peptide was incubated with purified recombinant PC2, a smaller peptide was generated. Analysis of CT peptide derivatives for their inhibitory potency revealed that CT peptide 1-18 (containing Lys-Lys at the carboxyl terminus) represented a potent inhibitor, but that peptide 1-16 was inactive. Inclusion of carboxypeptidase E (CPE) in the reaction greatly diminished the inhibitory potency of the CT peptide against PC2, in line with the notion that the CT peptide cleavage product is not inhibitory after the removal of terminal lysines by CPE. In summary, our data support the idea that PC2 cleaves the 7B2 CT peptide at its internal Lys-Lys site within secretory granules; deactivation of the cleavage product is then accomplished by CPE, thus providing an efficient mechanism for intracellular inactivation of the CT peptide.**

The eukaryotic subtilisin family of serine proteases is involved in the processing of prohormone and other precursor proteins through cleavage at paired or multiple basic residues (1–3). Prohormone convertase 2 (PC2), a member of this proteinase family, is believed to participate in the later stages of prohormone processing (4–7). It has recently been shown that the protein known as 7B2, whose expression is restricted to the central nervous system and to endocrine tissues (8–10), is intimately involved in proPC2 maturation (11, 12). This function appears to require the amino-terminal 21-kDa portion of the molecule (12). The portion of this protein (i.e., the last 31 amino acids of 7B2) that represents a potent inhibitor of PC2 and of the activation of immunopurified proPC2 (13, 14) is here termed the carboxyl-terminal (CT) peptide.

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The 31-amino acid CT peptide is known to represent a natural product of 7B2 biosynthesis (15, 16) and is cleaved from 27-kDa 7B2 within 50–60 min after initiation of synthesis (12, 17). It is believed, but has not yet been directly shown, that this peptide remains bound to proPC2—either as an inhibitor of zymogen activation or of PC2 activity itself—during transport through the secretory pathway. Because the CT peptide is present within neuroendocrine cells (15), this peptide could also conceivably function to inhibit active PC2 intracellularly. However, the mechanism for termination of inhibition is not yet understood. In an attempt to address this question, we have performed studies of the metabolic fate of the 7B2 CT peptide, as described below. We here present several lines of evidence, both *in vivo* and *in vitro*, to indicate that the 7B2 CT peptide can be further cleaved at an internal paired basic site, most likely by PC2 itself.

MATERIALS AND METHODS

Cell Lines, Metabolic Labeling, and Immunoprecipitation. An AtT-20 cell line stably expressing PC2 (6) was provided by R. E. Mains (Baltimore, MD). Rin5F cells were obtained from Gary Thomas (Portland, OR). Transfection of these cell lines has been described (12). αTC1–6 cells were a kind gift from Shimon Efrat (18). Samples (5×10^5 cells each) were labeled with 0.5 mCi of [^{3}H]valine (29 Ci/mmol; 1 Ci = 37 GBq; Amersham) in valine-deficient medium (prepared from a Select-Amine Kit; Life Technologies, Gaithersburg, MD). Steady-state labeling and pulse-chase experiments of the cells and immunoprecipitation of the cell extracts were performed as described (12). Cells were harvested by scraping into boiling buffer; the antiserum used for immunoprecipitations was raised against the carboxyl-terminal 13 amino acids of rat 7B2 (see below). Immunoprecipitates were resuspended in 200 μl of high pressure gel permeation chromatography (HPGPC) eluant (32% acetonitrile/0.1% trifluoroacetic acid) in the presence of 150 mM acetic acid and 100 μl subjected to HPGPC (see below).

RIA of Cell Extracts for CT-Related Peptides. *Peptides.* Except for 7B2 CT 1-18, which was synthesized by the Core Facility at the University of Chicago, peptides used as antigens and substrates were synthesized by either Genosys (The Woodland, TX) or the Louisiana State University Medical Center Core Laboratories; purity was verified by either mass spectroscopy or reverse-phase HPLC.

Antisera. To provide tools to follow the fate of the CT peptide, two different antisera were raised in New Zealand White rabbits against various portions of this peptide by Hazleton–JRH (Denver, PA). The first antiserum (23B6) was

Abbreviations: CT peptide, carboxyl-terminal peptide; PC1 and PC2, prohormone convertase 1 and 2; HPGPC, high pressure gel permeation chromatography; PMA, phorbol 12-myristate 13-acetate; CPE, carboxypeptidase E.

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raised against the last 13 amino acids (residues 174–186) of rat 7B2, with an amino-terminal tyrosine added for iodination purposes; succinylated hemocyanin was used as the carrier. The second antiserum (17B6) was raised against $h7B2_{155-170}$ (the first 16 amino acids of the human CT peptide; see Fig. 1) coupled to hemocyanin (Pierce). Rabbits were injected with ≈250 μg of conjugate every 3 weeks; the dose was dropped to 125 μg after the first two injections. Sera were obtained 10 days after each boost and were tested by determining ability to bind iodinated $Tyr\text{-}r7B2_{173-185}$ (antiserum 23B6) or $h7B2_{155-170}$ (antiserum 17B6) as described below.

Cell culture and extraction. RinPE-7B2 and AtT-20/PC2–7B2 cells were grown to 80% confluence in 10-cm dishes. They were then washed twice with 5 ml of Dulbecco's PBS and lysed in 1 ml of 1 M acetic acid/20 mM HCl/0.1% 2-mercaptoethanol. Following freezing and thawing, an aliquot of the homogenate was removed for the determination of total protein, and the remainder was centrifuged. The clear supernatant was lyophilized and redissolved in 250 μl of 32% acetonitrile/0.1% trifluoroacetic acid, and 100 μl was subjected to HPGPC in the same solvent as described above. Recovery of ^{125}I-labeled CT peptide added to homogenates and subjected to an identical procedure was 76–81%. Duplicate portions of each fraction were dried in the presence of 10 μg BSA and analyzed using the two RIAs against the 7B2 CT peptide. Each HPGPC experiment was verified by using an independent preparation of cells. For stimulation experiments, two plates of 80% confluent cells were rinsed in OptiMem (Life Technologies) twice, then incubated for 1 h (RinPE-7B2 cells) or 2 h (AtT-20/PC2–7B2 cells) at 37°C in OptiMem containing 100 μg aprotinin per ml/10 μg BSA per ml in the presence or absence of 100 nM phorbol 12-myristate 13-acetate (PMA). The medium was removed, centrifuged at low speed to remove floating cells, and stored frozen prior to concentration by Sep-Pak (Waters) chromatography. For the latter procedure, the thawed medium was acidified by the addition of 0.1% trifluoroacetic acid and applied to a conditioned Sep-Pak. The cartridge was rinsed with 10 ml of 1 M acetic acid, and the peptides were eluted with 6 ml of 60% 1-propanol/0.1% trifluoroacetic acid. The eluate was lyophilized and resuspended in 250 μl of 32% acetonitrile/0.1% trifluoroacetic acid before HPGPC. Size separation of peptides was performed by HPGPC as in ref.19.

RIAs. RIAs for $7B2_{155-170}$ (the amino terminal portion of the CT peptide, assay 17B6) and $7B2_{173-185}$ (the carboxyl terminus of the CT peptide, assay 23B6) were carried out in duplicate essentially as described (19). In brief, antisera, at a final dilution of 1:3000 for 17B6 and 1:6000 for 23B6, were incubated together with ≈10,000 cpm of peptide. For 23B6, the peptide $Tyr\text{-}r7B2_{174-186}$ was labeled (i.e., the last 13 amino acids of r7B2, preceded by an added Tyr); for 17B6, $h7B2_{155-170}$ (i.e., the first 16 amino acids of the human CT peptide) was labeled. Peptides were iodinated by the chloramine T method and purified by Sep-Pak reverse-phase chromatography. RIAs were carried out in a final volume of 300 μl RIA buffer (19) at 4°C overnight. Standards for the 23B6 assay ranged from 0.05 to 5 pmol $7B2_{174-186}$. Standards for the 17B6 assay ranged from 0.01 to 2.5 pmol $h7b2_{155-170}$. The sensitivity of the 23B6 assay (IC_{20}) was 0.11 ± 0.02 pmol, and the IC_{50} was 0.40 ± 0.01 pmol (mean ± SE, $n = 7$). The sensitivity of the 17B6 assay was 0.028 pmol and the IC_{50} was 0.104 pmol (mean ± SE, $n = 3$). Due to the substitution of three aspartates for glutamates in this portion of the peptide (see Fig. 1), human 1-31 CT peptide exhibits no cross reaction in the rat CT peptide assay (23B6). Because rat 1-31 CT peptide was not available, we were not able to estimate the relative potencies of intact versus truncated CT peptide derivatives in this assay.

Hydrolysis of ^{125}I-Labeled 7B2 CT Peptide by Purified PC2. 7B2 CT peptide was iodinated by the chloramine T method and 8000 cpm incubated with 0.28 μg purified recombinant mouse PC2 (33) in 100 mM sodium acetate buffer, pH 5.0 in the presence of 5 mM $CaCl_2$ and 0.2% Brij. After a 60-min incubation period at 37°C, samples were frozen for subsequent size separation by HPGPC.

Inhibition of des31,32 Proinsulin Cleavage by 7B2: Effect of Carboxypeptidase E. Five microliters of recombinant PC2 was incubated in a total volume of 50 μl in 0.1 M sodium acetate buffer, pH 5.0/0.1% Triton X-100/10 μM dithiothreitol/10 mM calcium chloride with various concentrations of 7B2 CT peptide, as indicated, and/or 0.25 μg of recombinant carboxypeptidase E (CPE) (provided by Lloyd Fricker, Albert Einstein College of Medicine). Following 30 min at 37°C, ^{125}I-labeled des31,32 human proinsulin (100,000 cpm) was added, and the incubation was continued for 30 min. Ten microliters of each of the reaction mixtures was resolved on a modified Tricine polyacrylamide gel (20), and the dried gel was subjected to autoradiography. Phosphoimage analysis was used to quantify the degree of conversion of des31,32 proinsulin to insulin.

Inhibition of PC2 by 27-kDa 7B2 and 7B2 CT Peptide Derivatives. Purified recombinant mouse PC2 (0.28 μg) was preincubated with varying concentrations of 27-kDa 7B2 (obtained by prokaryotic expression, as described in ref. 33), 7B2 CT peptide, 7B2 CT peptide 1-18, or 7B2 CT peptide 1-16 in 100 mM sodium acetate buffer, pH 5.0, in the presence of 5 mM $CaCl_2$ and 0.2% Brij at 37°C for 15 min. The fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-AMC (Cbz, benzyloxycarbonyl; AMC, aminomethylcoumarin) (21) was then added to 200 μM and the reaction continued for 72 min before the released fluorescent product aminomethylcoumarin was measured.

RESULTS

The 7B2 CT Peptide Is Cleaved Internally: Steady-State Labeling Studies. The CT peptide (Fig. 1) inhibits PC2 by binding to the active site of this enzyme (13, 14). To understand the mechanism of the termination of the inhibition–i.e., either dissociation or cleavage of this peptide–*in vitro* and *in vivo* experiments were performed. Since the CT peptide contains no methionines, but contains four valines, [^{3}H]valine was used to follow its biosynthetic pathway. In *in vivo* experiments, AtT-20/PC2–7B2 and RinPE/7B2 cells (12) as well as αTC1–6 cells (18), which represent the cell line richest in natural 7B2, were labeled with [^{3}H]valine in valine-deficient medium for 6 h; immunoprecipitation was carried out by using antiserum directed against the extreme carboxyl terminus of 7B2 (23B6) and peptides were separated by HPGPC. The results of such steady-state labeling experiments are shown in Fig. 2 *a–c*. A radioactive peak was observed at fraction 40–41; this elution position coincides with the position of synthetic human CT peptide. Furthermore, a smaller product, at fraction 45 (corresponding to a peptide of about 1.5 kDa) was also immunoprecipitated using the carboxyl-terminal antiserum from all three cell lines. This peptide possesses the correct molecular mass to represent the carboxyl-terminal portion of internally cleaved CT peptide—i.e., CT_{19-31} (see Fig. 1). It should be noted that the 1.5-kDa peptide contains only one valine as opposed to the four present in the parent molecule; the molar amount of this peptide relative to the CT peptide is thus underestimated by a factor of four. Radiosequencing of this peak confirmed the identity of this fragment (Fig. 2*d*).

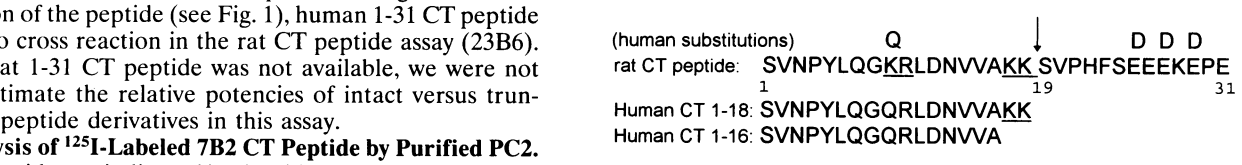

FIG. 1. Structure of the rat and human 7B2 CT peptides. Arrow depicts the site of cleavage by PC2.

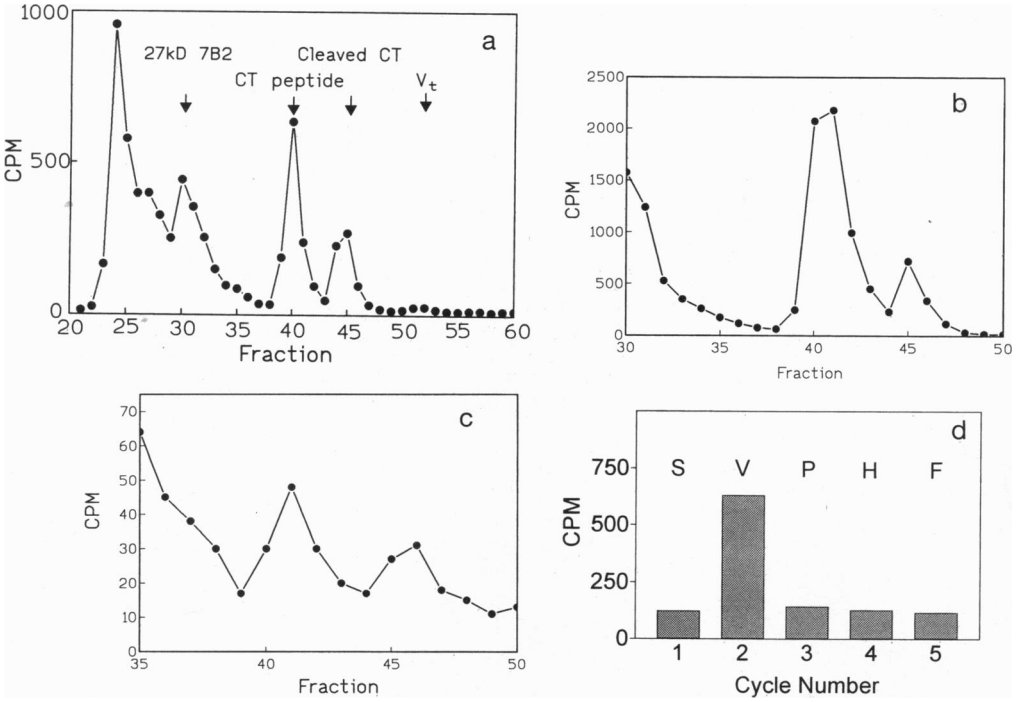

FIG. 2. Steady-state labeling of CT peptide-immunoreactive peptides in various cell lines. (*a*) AtT-20/PC2–7B2 cells. (*b*) RinPE-7B2 cells. (*c*) aTC1–6 cells. Cells were labeled for 6 h with tritiated valine; CT-immunoreactive peptides were immunoprecipitated using antiserum 23B6 and then size-fractionated by HPGPC. The void volume was at fraction 24. Note that there are four valines in the CT peptide and only one in the presumed cleavage product. Radiosequencing of the cleaved valine-labeled product, derived from AtT-20/PC2–7B2 immunoprecipitates (fractions 44–46), is depicted in *d*.

The CT Peptide Can Either Be Rapidly Secreted as the Intact Peptide or Slowly Cleaved Intracellularly: Metabolic Labeling Studies. The fate of the CT peptide was analyzed through pulse-chase experiments using AtT-20/PC2–7B2 cells. Cells were labeled with [^{3}H]valine in valine-deficient medium for 20 min, then chased for 30, 60, 120, 180, or 240 min. CT peptide-related peptides present in the cell extracts were immunoprecipitated with CT peptide antiserum (23B6), and immunoprecipitates were fractionated by HPGPC. The results, shown in Fig. 3, reveal a peak of radioactivity at the elution position of the intact CT peptide in all samples. In agreement with our previous study (12), we observed that even at the zero time point (immediately after the pulse) a significant amount of CT peptide was already cleaved from 27-kDa 7B2 (not shown). At 30 min of chase (Fig, 3) virtually all of the 27-kDa 7B2 was cleaved; this is consistent with immunoprecipitation results obtained using an antibody against 21-kDa 7B2, both employing [^{3}H]valine (data not shown) and [^{35}S]methionine labeling (12). The presence of a peak of radioactivity of smaller molecular mass indicates that the CT peptide is further cleaved at an internal site. Though this peak was not detectable at early times, it was distinguishable at 180- and 240-min chase times. The total CT peptide radioactivity of each chase time point was plotted against the chase time (not shown). The results indicate that the basal release of newly synthesized CT peptide from the cell is quite rapid, with about two-thirds occurring within the first 60-min chase period; because the peptide appears in the medium with similar kinetics, this release can probably be accounted for by constitutive secretion.

RIAs of AtT20/PC2 7B2 and RinPE-7B2 Cells Demonstrate the Existence of Cleaved CT Peptide Products. To provide further information on the CT peptide cleavage products, we developed two new RIAs, one against the extreme carboxyl terminus of the peptide (CT peptide assay) and one against the amino terminus of the CT peptide, corresponding to the carboxyl terminus of the expected cleavage product (1-16 assay). Fig. 4*a* shows the results of the CT peptide assay of an extract of AtT-20/PC2–7B2 cells. We found that ≈40% of the immunoreactivity eluted at a position consistent with that of the CT peptide, while 60% of the immunoreactivity eluted at the position of a smaller, about 1.5 kDa, peptide.

To determine the relative amount of CT peptide stored in regulated granules, AtT-20/7B2 cells were either stimulated with 100 nM PMA for 2 h or placed in medium lacking secretagogue; the medium was then concentrated via Sep-Pak chromatography, and the secreted peptides were separated using HPGPC. Fig. 4*b* shows the CT peptide immunoreactivity in the basal medium and in the stimulated medium. These data show a high basal rate of release of the CT peptide; in response to stimulation of the cells, the secretion of both the CT peptide as well as its carboxyl-terminal cleavage product were increased.

The same assays were also applied to RinPE/7B2 cells. Fig. 5 depicts the profile of immunoreactivity of the CT peptide in

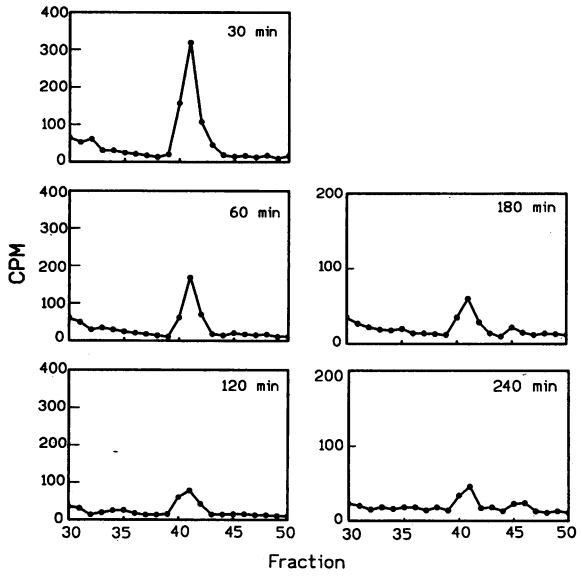

FIG. 3. Pulse-chase labeling of CT peptide-immunoreactive peptides in AtT-20/PC2–7B2 cells. Cells were labeled for 20 min with tritiated valine, and then chased for 30, 60, 120, 180, and 240 min; CT-immunoreactive peptides were immunoprecipitated using antiserum 23B6 and then size-fractionated by HPGPC. Background cpm were not subtracted.

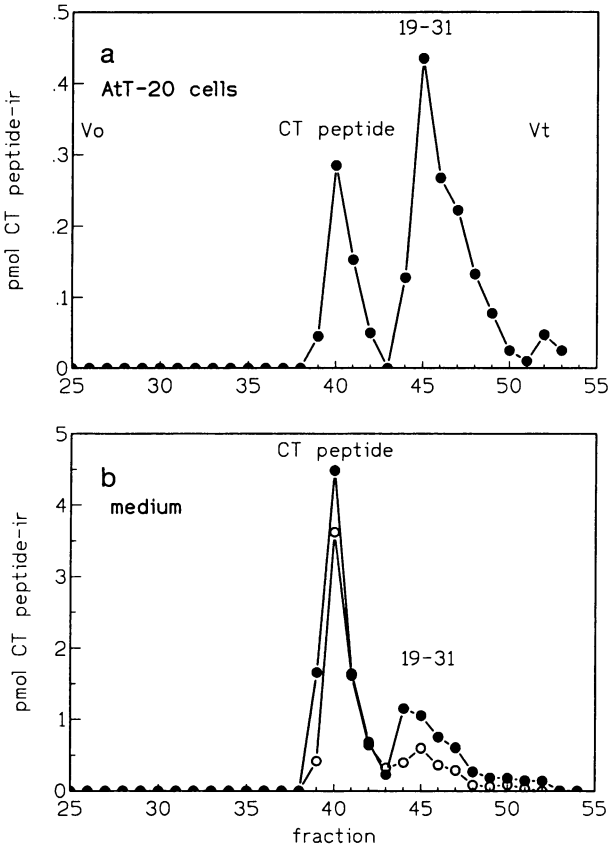

FIG. 4. RIA of CT peptide immunoreactivity in AtT-20/PC2–7B2 cells. Cells from a 10-cm dish of cells were homogenized in acid and centrifuged, and the supernatant was lyophilized. Following resuspension in HPGPC elution buffer and size fractionation, CT peptide-immunoreactive (ir) peptides were assayed in each fraction by radioimmunoassay. Results represent immunoreactivity per fraction in about one-third of the extract. (*a*) Cells. (*b*) PMA-stimulated medium (closed circles) and basal medium (open circles).

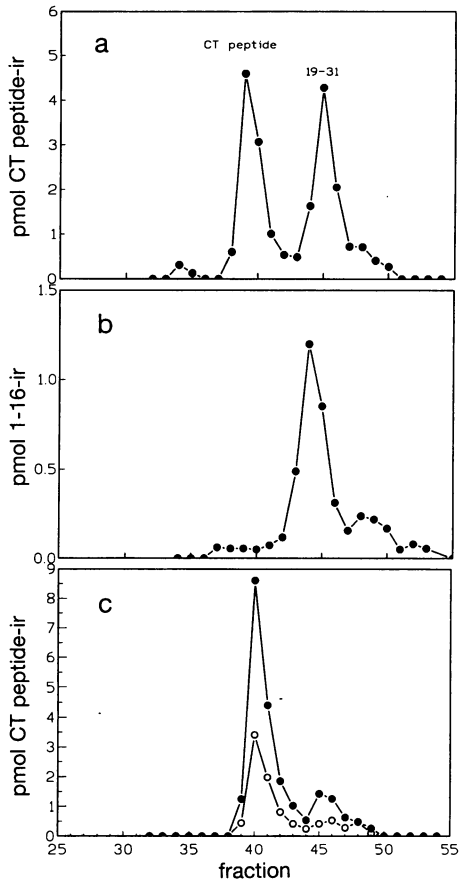

FIG. 5. RIA of CT peptide immunoreactivity in RinPE-7B2 cells. Cells from a 10-cm dish were homogenized in acid and centrifuged, and the supernatant was lyophilized. Following resuspension in HPGPC elution buffer and size fractionation, CT peptide-immunoreactive (ir) peptides were assayed in each fraction by RIA. Results represent immunoreactivity per fraction in about one-third of the plate. (*a*) Cells (CT peptide RIA). (*b*) Cells (1-16 peptide RIA of the same fractions as in panel *a*). (*c*) PMA-stimulated medium (closed circles) and basal medium (open circles).

cell extracts. This cell line apparently does not cleave the CT peptide quite as efficiently as the AtT-20/PC2–7B2 cell line; only about 40% cleaved peptide was observed (Fig. 5*a*). The fact of cleavage was verified by using the 1-16 assay, which demonstrated a large peak of immunoreactivity at the expected elution position (Fig. 5*b*). Interestingly, a later-eluting peak of immunoreactivity was also observed, potentially indicating further cleavage of the amino terminal portion of the CT peptide at the paired basic residues located at residues $K_{164}R_{165}$ in rat 7B2 (K_9R_{10} in the CT peptide; see Fig. 1). (The molar amounts of this peptide were not in agreement with those of the CT peptide; however, due to cost considerations this assay was developed against the human peptide rather than the rat, and thus the amount of the rat peptide is likely to be underestimated.) Stimulation of RinPE-7B2 cells with PMA resulted in the release of both the CT peptide and its carboxyl-terminal cleavage product (Fig. 5*c*). The potential further cleavage of the amino-terminal portion of the CT peptide was verified using the 1-16 assay which showed little basal release but pronounced stimulation of the release of both the amino-terminal peptide as well as a smaller putative cleavage product in the presence of the secretagogue (not shown). Taken together, these data indicate that both the CT peptide as well as fragments thereof are stored in regulated secretory granules.

Purified Recombinant PC2 Can Hydrolyze the 7B2 CT Peptide. We have previously shown that the interior Lys-Lys site is of key importance for the inhibition of PC2 by CT peptide derivatives (13, 14). To directly determine whether PC2 can cleave the CT peptide, the synthetic 7B2 CT peptide (labeled with ^{125}I) was incubated with purified recombinant PC2, followed by separation of products by gel filtration; a single product peak of radioactivity, possessing the appropriate mass to represent the amino-terminal tyrosine-containing product peptide, was observed, indicating the cleavage of the CT peptide by PC2 (Fig. 6). Reaction mixtures could be better resolved by using reversed-phase HPLC, which also yielded a single product peak of radioactivity (not shown).

7B2 CT Peptide Inhibits des31,32 Proinsulin Hydrolysis: CPE Effect. 7B2 CT peptide was also able to block the cleavage of radiolabeled des31,32 proinsulin by recombinant PC2 (Fig. 7*a*); about 50% inhibition was obtained at 20 nM 7B2 (quantitation presented in Fig. 7*b*). Interestingly, we found that inclusion of CPE in the reaction mixture was able to considerably diminish the inhibitory potency of the 7B2 CT peptide against PC2. CPE alone was not able to convert des31,32 proinsulin into insulin (not shown). These results led to the idea that a PC2 hydrolysis product, presumably CT peptide 1-18, remained inhibitory to PC2 until acted upon by CPE. Indeed, when synthetic CT peptide 1-18 was included in a reaction containing radiolabeled des31,32 proinsulin and recombinant PC2, it also proved to be highly inhibitory to PC2 (results not shown).

Relative Potencies of CT Peptide Fragments Against PC2. The inhibition potencies of 27-kDa 7B2, the CT peptide, and amino terminal fragments of the CT peptide against recom-

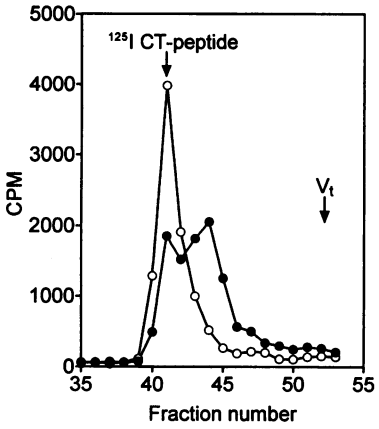

FIG. 6. Recombinant PC2 can cleave ^{125}I-labeled CT peptide. 7b2 CT peptide (160 nM final concentration) mixed with ^{125}I-labeled CT peptide was incubated with purified recombinant PC2 (2.8 μg) for 0 h (open circles) or 1 h (closed circles). The reactions were size-fractionated by HPGPC, and radioactivity in each was determined by γ spectroscopy.

binant purified PC2 were determined directly using the standard fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-AMC. In agreement with previous results using immunopurified PC2 (14, 22), we found that both 27-kDa 7B2 and the CT peptide represented very potent inhibitors of recombinant PC2 (IC_{50} = 14 ± 2 nM and 282 ± 3 nM, respectively) (Fig. 8). Further, in support of the results obtained using proinsulin hydrolysis, the truncated CT peptide 1-18 (terminating in Lys^{17}-Lys^{18}) was also found to represent a powerful inhibitor of PC2, with half-maximal inhibitory potency of 272 ± 1 nM (mean ± SEM, 3 replicates). However, CT peptide 1-16 (lacking the terminal Lys-Lys) displayed little inhibitory effect even at extremely high concentrations (Fig. 8).

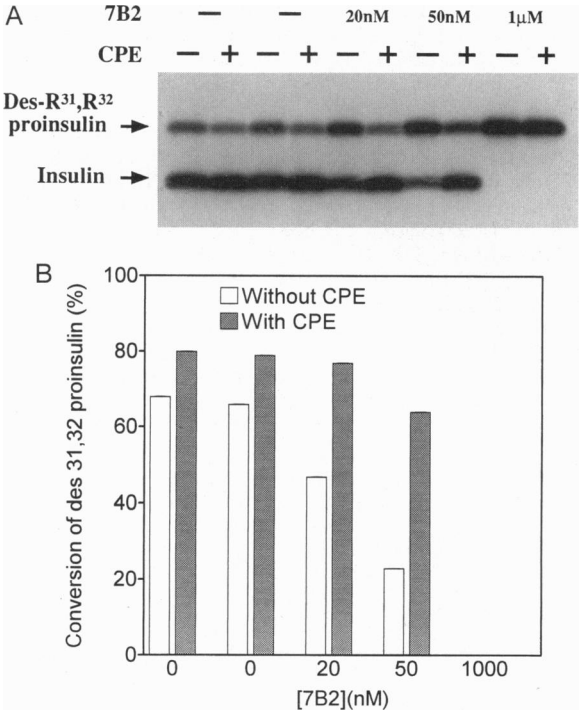

FIG. 7. CT peptide inhibits PC2-mediated cleavage of radiolabeled des31,32 proinsulin; CPE reduces the potency of the CT peptide. (*a*) Autoradiograph of a polyacrylamide gel of ^{125}I-labeled proinsulin incubated with recombinant PC2 in the presence of various amounts of the CT peptide, with or without CPE. (*b*) Quantitation of the phosphoimage.

DISCUSSION

The family of eukaryotic subtilisin-like enzymes has grown to at least seven forms at present (23). However, very little is known as to how the activity of these enzymes is regulated under physiological conditions. It is clear that mature, active enzymes in this family are obtained by proteolytic processing of precursors by cleavage of the proregion. The conversion of proPC2 to mature PC2 has been shown to be a slow process, occurring late in the secretory pathway, and is most likely autocatalytic (24–26, 33).

Recent reports have implicated the neuroendocrine protein 7B2 in the biosynthesis of active PC2, both in the maturation of PC2 and in the regulation of its activity (11–14, 32). Our previous studies have revealed that the 7B2 CT peptide, which represents a natural product (15), is a potent PC2 inhibitor (14). In this report we have investigated the mechanism of termination of PC2 inhibition. Our results confirm the intracellular production of the CT peptide by neuroendocrine cells, and additionally demonstrate that the CT peptide can be further cleaved intracellularly. Steady-state labeling experiments in three different cell lines revealed the existence of CT peptide cleavage products, and these results were independently corroborated using two new RIAs directed against this peptide. The stimulated secretion experiments revealed that both intact CT peptide as well as its cleaved products are stored in regulated secretory granules. Interestingly, these results also indicated that one of the CT peptide cleavage products is itself further cleaved, presumably at an independent paired basic site (residues 9–10). This pair of basic residues is present only within rat 7B2, although a single basic residue at this site is conserved among all species examined to date (27).

Given that the site of binding of the CT peptide to PC2 has been shown to be localized to Lys^{17}-Lys^{18} (13, 14), the proteinase most likely to be responsible for the interior cleavage of the CT peptide is PC2 itself. This idea was also supported by experiments showing lack of CT peptide cleavage in non-PC2-expressing AtT-20/7B2 cells (results not shown). Cleavage of protein inhibitors by the proteinase under inhibition is a common finding in enzyme inhibition, with cleaved inhibitor products retaining full inhibitory potency in relatively stable enzyme-inhibitor complexes (28). A recent example of this phenomenon is the inhibition of interleukin 1β converting enzyme family members by baculovirus antiapoptotic protein p35 (29). Our *in vitro* assays directly demonstrated that purified recombinant PC2 could in fact cleave iodinated CT peptide. Given the specificity of PC2, the most likely site for CT peptide

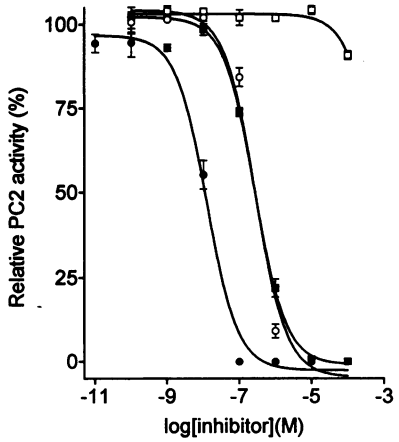

FIG. 8. Inhibition of recombinant PC2 by synthetic CT peptide-derived peptides. Various concentrations of CT peptide-derived peptides were assayed for their inhibitory potency against PC2. Closed circles, recombinant rat 27-kDa 7B2; open circles, CT peptide 1-31; closed squares, CT peptide 1-18; open squares, CT peptide 1-16.

cleavage is following the Lys-Lys pair, resulting in the production of two fragments, 1–18 and 19–31 (see Fig. 1). This cleavage site was confirmed by radiosequencing. An interesting finding was the fact that the inhibitory potency of the CT peptide was considerably diminished by the inclusion of CPE in the reaction mixture. It is well known that CPE is a highly efficient enzyme in cleaving basic amino acid residues from the carboxyl termini of peptides within secretory granules (23, 30). These results suggested that separate enzymatic action by CPE was required to abolish the inhibitory potency of the CT peptide, and this notion was confirmed when we directly tested the relative potencies of CT peptide products containing or lacking the terminal Lys-Lys against recombinant PC2. We found that the 1-18 peptide was still extremely inhibitory to PC2, but that the 1-16 peptide completely lacked inhibitory activity. The action of CPE thus appears to be vital to the termination of inhibition of the CT peptide.

Based on the data presented above, we propose the following model for the metabolism of the CT peptide. Quite soon after synthesis (even within the 20-min labeling period) (12), the intact CT peptide is cleaved from 27-kDa 7B2, most likely within the Golgi apparatus (17). The CT peptides which are sorted to the secretory granule compartment are gradually cleaved after Lys^{17}-Lys^{18} by PC2, and this lysine-extended peptide remains inhibitory to PC2 in the PC2–CT peptide complex. Following slow dissociation of CT peptide 1-18 from the complex, CPE acts to remove the terminal lysines from the cleaved CT peptide, thus inactivating this inhibitor. The fact that secretory granules contain relatively high concentrations of intact CT peptide implies that this peptide may continue to serve a regulatory role with respect to PC2 within this subcellular compartment. Our results may be relevant to the puzzling accumulation of proinsulin in the fat/fat mouse, which lacks functional CPE (31), in that high levels of Lys-Lys-extended CT peptide could act to block the action of endogenous PC2 within secretory granules.

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