Prohormones are known to be processed at various cleavage sites in a defined temporal order, suggesting the possibility of sequential unfolding of processing sites. In order to investigate whether sequential processing at predefined sites is in fact required for proper processing, site-directed mutagenesis was performed to block known initial cleavage sites within proenkephalin. Pulse-chase/immunoprecipitation experiments were employed to analyze the fate of mutant and native proenkephalins in stably transfected AtT-20 cells. While processing did not occur at blocked sites, surprisingly, overall processing of mutant proenkephalins proceeded efficiently, and alternative sites were chosen. When compared with native proenkephalin, processing of mutant proenkephalins occurred more slowly at early stages and more quickly at later stages. Experiments employing endoglycosidase H indicated that the early slow processing of mutant proenkephalins may be due to delays in intracellular transport. Metabolic labeling studies showed that more efficient production of bioactive opioids occurred in all processing site blockade mutants examined; these results were confirmed using several different radioimmunoassays of stored peptide products. We conclude that efficient processing of prohormone precursors does not require a specific temporal order of processing events. The fact that mutant proenkephalins were more fully processed than native proenkephalin may provide a route for more efficient production of opioid peptides in applications for chronic pain treatment.

In endocrine and neuronal cells, peptide hormones and pro-neuropeptides are synthesized as large precursor proteins that are then endoproteolytically processed during intracellular transport to produce the biologically active molecules. After posttranslational modification, these precursors are sorted in the trans-Golgi network and packed into dense core secretory vesicles for storage until release upon stimulation (1, 2). Common recognition sites present in these proproteins are pairs of basic amino acid residues, such as the preferentially cleaved Lys-Arg and Arg-Arg sites, and less frequently Lys-Lys and mon recognition sites present in these proproteins are pairs of vesicles for storage until release upon stimulation (1, 2). Com-

1 The abbreviations used are: PC1, prohormone convertase 1; PC2, prohormone convertase 2; HPGPC, high pressure gel permeation chromatography; -ir, immunoreactivity; enk, enkephalin; PE, proenkephalin; RIA, radioimmunoassay; enk, endoglycosidase H; I1 and I2, intermediate peptides.

Processing Site Blockade Results in More Efficient Conversion of Proenkephalin to Active Opioid Peptides*

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Pulse-Chase Experiments and Immunoprecipitations—For the pulse-chase experiments, subconfluent 35-mm wells (1–2 × 10^6 cells) of native PE-expressing and PE blockade mutant cell lines were subjected to the labeling procedure essentially as described previously (25). In brief, for the 20-min pulse labeling, 1 ml of methionine-free medium containing 1 μCi of [35S]methionine (average specific activity >1100 Ci/mmol, Amer sham Corp.) was added to each well. Cells were then chased for 0, 0.5, 1, 2, or 4 h. After each chase incubation, cells were scraped into 1 ml of ice-cold 1 x acetic acid, 20 mM HCl, and 0.1% (v/v) β-mercaptoethanol (solution A). Cell extracts were frozen and thawed, and insoluble material was removed by centrifugation. Samples were then subjected to a Met-enk-Arg-Phe radioimmunoassay as described previously (25). The highest expressing clones of each cell line were selected for further study.

Radiosequencing—In order to identify the metabolic intermediate eluting at fraction 35 in the K1 cells, a subconfluent 35-mm well of this cell line (approximately 1–2 × 10^6 cells) was used for labeling and immunoprecipitation experiments. One ml of methionine-deficient medium (Amer sham) containing 1 μCi of [35S]methionine was added, and cells were incubated at 37°C in an atmosphere containing 5% CO2 for 6 h. The immunoprecipitation procedure followed was as described above, using a combination of Xandra antisera (8) and JAS antisera (Met-enk-Arg-Phe) (28). Immunoprecipitated peptides were separated by HPGPC. A major peak of radioactivity, which had an apparent molecular size of 5 kDa, was pooled and subjected to automated Edman degradation (performed by the San Diego State University Microchemical Core Facility). Each cleaved residue was collected for liquid scintillation counting. Radiosequencing was performed only once.

RIA—Cells from each native and mutant PE-expressing clone were counted and 2.6 × 10^6 cells grown for 2 days in 10-cm Petri dishes. Duplicate 5-μl diluted (1:10) samples of the clarified cell extracts were subjected to RIA analyses to determine the overall proenkephalin expression levels.

Materials and Methods

Mutagenesis of Proenkephalin and Construction of the Expression Vectors—Rat proenkephalin cDNA (20) was excised from the plasmid pEV/RENK (21) and ligated into pRcCMV (+) (Invitrogen) as described previously (8). Three individual mutations and one double mutation of paired basic residues of PE were performed as shown in Fig. 1. The first individual mutation altered Lys237 to His237 and Arg238 to Lys238 and was termed K1. The second individual mutation converted Lys196 to His196 and Arg197 to Lys197 and was designated K2. The third individual mutation involved the alteration of Lys184 to His184 and was called K3. The double mutation, named K4, contained the same mutations as K1 and two additional substitutions, Lys178 to His178 and Arg179 to Lys179. The mutations were carried out by the Kunkel method (22) and were confirmed by dideoxy DNA method using standard methods.

Cell Culture and DNA Transfection—AtT-20/dv16 cells (obtained from R. E. Mains and B. A. Eipper) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 2.5% fetal bovine serum (prepared by the Kunkel method) (22) and were confirmed by dideoxy DNA method using standard procedures (23). Before use in transfection procedures, plasmids were purified twice by centrifugation through CsCl gradients.

Opioid peptides, a synthetic 5.2 kDa molecule, was used for quantitation by phosphoimaging analysis (Bio-Rad). The opioid peptides were labeled with [3H]-GTP or [35S]methionine, and the [3H]-GTP labeled peptide was used to determine the overall proenkephalin expression level (23, 25). Immunoprecipitated peptides were separated through CsCl2 gradients.

In order to identify the metabolic intermediate eluting at fraction 35 in the K1 cells, a subconfluent 35-mm well of this cell line (approximately 1–2 × 10^6 cells) was used for labeling and immunoprecipitation experiments. One ml of methionine-deficient medium (Amer sham) containing 1 μCi of [35S]methionine was added, and cells were incubated at 37°C in an atmosphere containing 5% CO2 for 6 h. The immunoprecipitation procedure followed was as described above, using a combination of Xandra antisera (8) and JAS antisera (Met-enk-Arg-Phe) (28). Immunoprecipitated peptides were separated by HPGPC. A major peak of radioactivity, which had an apparent molecular size of 5 kDa, was pooled and subjected to automated Edman degradation (performed by the San Diego State University Microchemical Core Facility). Each cleaved residue was collected for liquid scintillation counting. Radiosequencing was performed only once.

RIA—Cells from each native and mutant PE-expressing clone were counted and 2.6 × 10^6 cells grown for 2 days in 10-cm Petri dishes. Duplicate 5-μl diluted (1:10) samples of the clarified cell extracts were subjected to RIA analyses to determine the overall proenkephalin expression levels.
pression level in each clone. In addition, duplicate aliquots of each fraction obtained from the HPGPC (as described above), were vacuum-dried in polypropylene tubes in the presence of bovine serum albumin as carrier protein. Fractions were resuspended in 100 ml of RIA buffer (0.1 M sodium phosphate, pH 7.4, containing 0.1% heat-treated bovine serum albumin, 50 mM sodium chloride, 0.1% sodium azide, and 0.1% b-mercaptoethanol) and subjected to RIA as described previously (26). Briefly, specific antiserum, sample or standard, and 10,000 cpm of 125I-labeled peptide (Amersham) were incubated overnight at 4°C. The antisera used for RIA were raised against Met-enk-Arg-Phe (JAS antiserum) as described in Ref. 28, against Met-enk-Arg-Gly-Leu (29), or against Met-enk (RB4 antiserum; Ref. 30). Carrier γ-globulin and 25% polyethylene glycol were added to precipitate the bound label, which was separated by centrifugation. Radioactivity in pellets was determined using an LKB g-counter. RIA sofall cell lines were carried out at least four times on independent preparations of cells with similar results.

RESULTS

In order to examine the effect of site blockade on the processing of PE, four mutant cell lines were constructed. Fig. 1 depicts a diagrammatic representation of PE showing the different sites of mutation and the peptides known to be present in bovine adrenal medulla (9) are shown below (rat and bovine pre-PE have identical cleavage sites): Met-enk-Arg-Phe (Met-enk-RF), Met-enk, Met-enk-Ang-Gly-Leu (Met-enk-RGL), and Leu-enk. Lys237 and Arg238 were mutated to His237 and Lys238 (K1), Lys210, Arg211, Lys237, and Arg238 were mutated to His210, His211, His237, and Lys238 (K4). Lys196 and Arg197 were mutated to His196 and Lys197 (K2). Lys141 was mutated to His141 (K3). The asterisk indicates the location of the altered cleavage site for K1.

Fig. 1. Biosynthetic pathway of processing of PE and sites of mutations for blockade mutants. Peptides known to be present in bovine adrenal medulla (9) are shown below (rat and bovine pre-PE have identical cleavage sites): Met-enk-Arg-Phe (Met-enk-RF), Met-enk, Met-enk-Ang-Gly-Leu (Met-enk-RGL), and Leu-enk. Lys237 and Arg238 were mutated to His237 and Lys238 (K1), Lys210, Arg211, Lys237, and Arg238 were mutated to His210, His211, His237, and Lys238 (K4). Lys196 and Arg197 were mutated to His196 and Lys197 (K2). Lys141 was mutated to His141 (K3). The asterisk indicates the location of the altered cleavage site for K1.

Fig. 2. Biosynthetic pathway of Met-enk-Ang-Phe-ir peptides in native PE and K1 cells shows a slower processing rate at initial steps and a faster cleavage into active opioids. Subconfluent 35-mm wells (1–2 × 106 cells) of native (A) and K1 (B) cells were incubated with 1 mCi/well [35S]methionine for 20 min. Cells were chased for 0, 0.5, 1, 2, or 4 h, and extracts were immunoprecipitated with antisera Xandra. Immunoprecipitates were size-fractionated by HPGPC, and the radioactivity in each fraction was quantitated by liquid scintillation spectroscopy. Arrows indicate the position of the size standards used: thyroglobulin (Vo), [35S]proenkephalin (PE), Peptide B (B), Met-enk-Ang-Phe (H), and salt volume (Vt).

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In order to examine the effect of site blockade on the processing of PE, four mutant cell lines were constructed. Fig. 1 depicts a diagrammatic representation of PE showing the different sites of mutation and the peptides known to be generated from the precursor molecule.

The Single Blockade Mutant K1 Is Processed First into an Intermediate of Unknown Identity, Which Is Subsequently Rapidly Processed to Met-enk-Ang-Phe—To investigate the effect of site blockade processing in PE in AtT-20 cells, pulse-chase experiments were carried out on cells expressing native PE and a single blockade mutant (at the initial cleavage site; K1 in Fig. 1). Fig. 2 shows the fate of the Met-enk-Ang-Phe portion of native and mutant PEs. For these experiments, immunoprecipitation was performed using antisera directed against Met-enk-Ang-Phe, the carboxyl-terminal peptide of PE (this antisera recognizes all peptides terminating in this heptapeptide). A single peak, corresponding to the approximate elution position of PE, was observed in both cell lines after a 20-min pulse. Native proenkephalin was initially processed more quickly than the K1 mutant (Fig. 2, compare panels A and B at 0.5 h of chase), as shown by a stronger decrease in the peak size of PE relative to 0 h as well as the presence of a processing product in native PE, which was not observed in the K1 mutant at this time. The K1 mutant instead exhibited an intermediate peak of unknown identity (designated as I1; fraction 35, approximately 5 kDa) and a small peak that corresponded to the elution position of the Met-enk-Arg-Phe standard (fraction 45). At 1 h of chase, conversion of Peptide B to Met-enk-Arg-Phe was evident in native PE. The final molar ratio of Peptide B to Met-enk-Arg-Phe was 2.5:1 in this cell line. By comparison, the K1 mutant still contained intact PE and exhibited a 0.1:1 molar ratio of I1 to Met-enk-Arg-Phe, indicating a faster processing to active PE-derived opioids at 1 h of chase.

Similar results to the above were observed at the 2-h chase time. Two peaks corresponding to Peptide B and Met-enk-Arg-Phe, at a molar ratio of 1:1, were observed in native PE (Fig. 2A; note that two methionines are present in Peptide B but only one in Met-enk-Arg-Phe). At this time, about 40% of the total radioactivity in the immunoprecipitate was present as Met-enk-Arg-Phe in the mutant PE, as compared with only about...
K1 cells were incubated with 1 mCi of [35S]methionine for 6 h, extracted, generated by cleavage on the carboxyl side of Lys210-Arg211. The enk-Arg-Phe antiserum and the molecular size of the peptide, the ability of the peptide to be immunoprecipitated with Met-enk-Arg-Phe antiserum, we speculate that this peak is a product of alternative cleavage at the carboxyl side of Lys210-Arg211. This extended form of Peptide B is indicated by an asterisk in the PE diagram (Fig. 1). The radiosequencing results thus suggest that an alternative site, but in fact not the nearest paired basic site, was used for initial cleavage when the naturally preferred site was unavailable.

**The Double Blockade Mutant PE (K4) Is Also Processed at a Faster Rate during Initial Cleavage Stage and Gives Rise to an Alternative Intermediate Peptide**—Since the processing of PE was apparently not hampered by a mutation at the initial processing site, we decided to blockade the newly chosen site (Lys210-Arg211) as well in the K1 mutant (Fig. 1). This new PE mutant, termed K4, was subjected to a similar pulse-chase and immunoprecipitation experiments using Met-enk-Arg-Phe antiserum. Eluates from immunoprecipitates were size-fractionated by HPGPC. Arrows indicate the position of the size standards used: thyroglobulin (V0), [35S]proenkephalin (PE), Peptide B (B), Met-enk-Arg-Phe (H), and salt volume (Vf).

With Met-enk-Arg-Phe antiserum, we speculated that this peak is a product of alternative cleavage at the carboxyl side of residues Arg217-Arg218. Following 1 h of chase, the molar ratio of Peptide B to Met-enk-Arg-Phe was 2.7:1 in native PE. In contrast, the molar ratio of I2 Met-enk-Arg-Phe was 0.3:1 in K4 at the chase time. These results indicate that processing of I2 to Met-enk-Arg-Phe was more extensive in K4 PE than the analogous processing event in native PE (Fig. 4). At 2 and 4 h of chase, this same trend continued, with higher Met-enk-Arg-Phe production in the K4 mutant PE as compared with native PE. At 4 h of chase, the molar ratio of Peptide B to Met-enk-Arg-Phe in native PE was 0.8:1; however, in the K4 mutant the intermediate peptide had been completely converted to Met-enk-Arg-Phe. In summary, these results demonstrate that like the K1 mutant, K4 mutant PE was processed more slowly at initial steps, but more rapidly and more completely at later steps, than was native PE. Thus blockade of processing at two of the preferred initial processing sites also did not halt proteolytic cleavage but instead resulted in a shift of cleavage to yet another site. Similar kinetic of processing were observed in pulse-chase and immunoprecipitation experiments using two additional clones from the K4 mutant expressing either comparable or lower levels of proenkephalin.

**Endoglycosidase H Sensitivity in the K4 Mutant PE Indicates Longer Retention in the Endoplasmic Reticulum**—The meta-
bolic labeling studies above indicated that the initial stages of PE processing were slower in cells containing mutant forms of PE. In order to examine if this could be a result of slower arrival at processing compartments, i.e. longer retention in the endoplasmic reticulum, we subjected double mutant K4 and native PEs to endo H and immunoprecipitated proteins were subjected to SDS-PAGE to estimate the appearance of endo H-resistant forms. Fig. 5 shows the profile of endo H sensitivity of radiolabeled PE at different time points after synthesis. At 0 h of chase, native PE and K4 mutant PE showed two bands, corresponding to comparable amounts in the two cell lines of glycosylated and unglycosylated PE forms. At this time point, glycosylated PEs in both mutant and native cell lines were completely endo H-sensitive. After 20 min of chase, the glycosylated native PE began to exhibit some endo H resistance, while the glycosylated mutant PE was still almost completely endo H-sensitive. In addition, both PE bands in native PE were less intense when compared with the double mutant, indicating a loss of native PE by further processing. This provided confirmation of the pulse-chase analyses in which native PE was processed at a higher rate at initial stages (Fig. 4A). Following 40 min of chase, endo H-treated native PE showed an approximately equal ratio of glycosylated to unglycosylated PE (seen best in a longer exposure of the autoradiograph), indicating that the glycosylated form had acquired complete endo H resistance. At this time, the K4 mutant PE still showed some endo H sensitivity (Fig. 5). In addition, a lower molecular weight band had appeared in native PE, corresponding to Peptide B, and in the double mutant, representing I. At 60 min of chase, about half of the glycosylated form of the mutant PE still remained endo H-sensitive; intact native PEs were almost completely processed. These results provide evidence that the mutant PE takes longer to arrive at the medial Golgi compartments (where resistance to endo H is achieved) than native PE, most probably due to longer residence in the endoplasmic reticulum.

Processing of PE in Initial K1 Blockade Mutants Results in More Efficient Production of Active Opioid Peptides—To confirm the efficient processing of mutant PEs into active opioids, additional pulse-chase labeling and immunoprecipitation experiments were performed using native PE and the PE and mutant K1 cell lines employing antiserum against another part of the proenkephalin precursor, Met-enkephalin (Met-enk). The antiserum recognizes peptides terminating in this pentapeptide but recognizes the PE precursor very poorly; therefore, only 2- and 4-h chase point periods are presented. Fig. 6 depicts the data for native PE (panel A) and initial blockade mutant, K1 (panel B). The peaks observed in both cell lines at 2 h of chase corresponded to the approximate elution positions of the 8.6-kDa peptide, Peptide F, and Met-enk. Following 4 h of chase, the amounts of the 8.6-kDa peptide and Peptide F were diminished in both cell lines. The molar ratios of Peptide F to Met-enk were 1:9 in native PE and 1:13 in K1 mutant PE. Thus, the main difference between these two cell lines was the presence of a higher amount of Met-enk in the mutant PE cell line (panel B) when compared with the native PE-expressing cell line (panel A). These results support the findings obtained with the Met-enk-Arg-Phe antiserum in demonstrating an increased extent and rate of processing of K1 mutant PEs as opposed to native PE.

K2 and K3 Mutant PEs Are Also More Slowly Converted to Peptide B and More Rapidly Converted during the Peptide B to Met-enk-Arg-Phe Step than Native PE—In order to determine whether the extensive processing of PE in the K1 and K4 mutant cell lines was site-specific, we constructed mutant PEs blockaded at other processing sites (Fig. 1). Figs. 7 and 8 both show results from the Met-enk-Arg-Phe immunoprecipitates of native PEs and K2 and K3 (panels A and B) mutant PEs. After the 20-min pulse, a peak corresponding to the position of the PE standard was observed in all cell lines, indicating efficient expression of mutant PEs. After 0.5 h of chase, Peptide B was
present in all cell lines (Figs. 7 and 8). However, at this time, only about one-third of the total sample radioactivity was present in intact native PE, while about one-half or more was present in intact mutant PEs (Figs. 7 and 8, compare panels A and B), indicating slower initial processing of mutant PEs. At 1 h of chase, some mutant PE still remained unprocessed, unlike native PE. At 2 h of chase, the amount of radioactivity in Peptide B compared with Met-enk-Arg-Phe was higher in native PE as compared with the mutant K2 and K3 PEs, indicating faster processing into Met-enk-Arg-Phe in the latter. These results were confirmed at the 4-h chase time. Thus, these PE mutants exhibited the same profile of processing as the previous two tested, i.e. depressed initial rates of processing and enhanced later rates. Comparable results were obtained during two different sets of experiments using both K mutants and another K2 clone expressing a similar level of PE.

**RIA of Stored Peptides in Mutant Cell Lines Supports the Finding of More Extensive Processing of PE into Active Opioids**—To determine the profile of stored immunoreactive enkephalins in mutant and native cell lines, RIAs were performed on gel filtration fractions of cellular extracts. The Met-enk-Arg-Phe RIA revealed two peaks of immunoreactivity, Peptide B and Met-enk-Arg-Phe, in extracts from cell lines expressing native PE; however, only one peak, Met-enk-Arg-Phe, was present in all four mutant cell lines (Fig. 9A). Fig. 9B shows the results from the RIAs using antiserum against Met-enk-Arg-Gly-Leu as described previously (8). Native PE was processed into one significant Met-enk-Arg-Gly-Leu-ir peak. However, the K1, K3, and K4 blockade mutants showed two peaks, corresponding to the 5.3-kDa PE-derived peptide and Met-enk-Arg-Gly-Leu. K3 mutants are not shown, since blockade at this site resulted in no Met-enk-Arg-Gly-Leu-ir peptide production. These RIA results support the pulse-chase analyses of mutant cell lines in demonstrating more efficient processing of the PE blockade mutants relative to native PE. The overall PE expression levels were determined in each clone and expressed as total Met-enk-Arg-Phe immunoreactivity per 10-cm plate, as follows: native PE (73 pmol/plate), K1 (440 pmol/plate), K2 (623 pmol/plate), K3 (258 pmol/plate), K4 (250 pmol/plate). Because of the more extensive processing in mutant PE clones as compared with native PE, and because free Met-enk-Arg-Phe reacts better in the Met-enk-Arg-Phe assay than does Peptide B, these values should be treated with caution. Western blot analyses using antibodies against PC1 indicated that the expression level of this enzyme was comparable in all four mutants and in the native PE-expressing clones.2

**DISCUSSION**

The posttranslational processing of proenkephalin has been examined in various types of cells, such as bovine adrenal medullary chromaffin cells (29–31) and PE-transfected AtT-20 (8, 25) and rat insulinoma Rin5f cell lines (25). Results from these studies indicate that processing of PE begins at the

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2 K. Johanning and I. Lindberg, unpublished data.
Arrows indicate the elution positions of synthetic standards.

carboxyl-terminal side of the molecule, producing Peptide B.

Subsequently, other cleavages such as the cleavage at Lys^196-
Arg results in intermediate sized peptides that will become
processed at later stages, eventually producing the bioactive
penta- to octa peptides. Very little is known about the biochemical
basis for the observation of temporally ordered cleavage of
prohormone precursors at specific sites (reviewed in Ref. 1).
Rhodes et al. (19) have provided evidence for sequential unfold-
ing of the proinsulin molecule such that cleavage at one site
provides a more favorable substrate for the action at another
site.

In the work reported here, we have employed site blockade of
the initial cleavage sites of PE in order to examine whether or
not processing of this precursor to the mature form must occur
in a predefined order. Contrary to our expectations, processing
profiles of these mutant PEs indicated that cleavage need not occur in a specific order. Our data demonstrated that all mu-
tant forms of PE were efficiently expressed and processed and
that alternative initial cleavage sites were utilized when the
preferred sites were unavailable. These results are reminiscent
of those obtained with Aplysia egg-laying hormone in AtT-20
cells (16). In this study, removal of an initial tetrabasic proc-
ressing site by deletion resulted in alternative cleavage at a
tribasic site on eventual efficient production of egg-laying hor-
mone (16). Wilson and co-workers (32) have recently presented
data on the processing of human PE to larger intermediates in
bovine chromaffin cells using Western blotting and monoclonal
antibodies. In this study, human PE was mutated at 12 dibasic
processing sites by converting Lys-Arg sequences to Lys-Gln,
and Arg-Arg sites to Arg-Gln. At four of these cleavage sites,
blockade of processing was achieved, and while none of the
usual processing products were produced, proenkephalin did
not remain intact but was processed into unnatural interme-
diates. Thus, for at least two different precursors, a major
alteration of cleavage site usage appears to result in efficient
enzyme processing of alternative sites and processing via un-
natural intermediates.

While processing of proenkephalin to end products proceeded
without hindrance in mutant PEs, our results clearly demon-
strate differences in the rate and extent of processing of mutant
PEs as compared with native PE. Initial processing was slowed
in all mutant PEs examined, as judged from the disappearance
of intact PE, the production of initial intermediates, and the
acquisition of endo H resistance. Since the introduction of
mutations may be expected to alter the normal conformation of
the precursor, we speculate that these altered conformations
are recognized as unnatural by quality control mechanisms in
the endoplasmic reticulum, potentially resulting in a delay in
export to the trans-Golgi network. However, once mutant PEs
reached the Golgi and underwent initial cleavage at alternative
sites, processing of aberrant intermediates appeared to occur at
an enhanced rate than with native PE.

Perhaps the most interesting aspect of the processing of
these mutant PEs is the extent of processing of the aberrant
intermediates to the penta- and heptapeptide bioactive en-
kephalins. Both metabolic labeling and RIAs indicated a much
greater generation and storage of mature enkephalins in all
four mutant PE cell lines as compared with the native PE
expressing cell line. Overexpression of PC1 relative to proop-
1S

Fig. 9. Radioimmunooassay of stored enkephalins confirms en-
36
hanced production of mature enkephalins in mutant cell lines.
A cell extract was prepared from a subconfluent 10-cm Petri dish by
37
38
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homogenization in solution A and clarified by centrifugation. Aliquots
31
of this extract corresponding to each cell line were size-fractionated by
HPGPC. Duplicate aliquots of each fraction were dried under vacuum,
of which lead to the production of unnatural intermediates, are
selected when preferred sites are unavailable; 2) initial proc-
sessing of site-blockaded proenkephalin is slowed, potentially by
longer residence in the endoplasmic reticulum and by the neces-
sity to use alternative sites; 3) unnatural intermediates are

In summary, we conclude that 1) processing of proenkepha-
lins does not require a predefined order, since alternative sites,
which lead to the production of unnatural intermediates, are
selected when preferred sites are unavailable; 2) initial proc-
sessing of site-blockaded proenkephalin is slowed, potentially by
longer residence in the endoplasmic reticulum and by the neces-
sity to use alternative sites; 3) unnatural intermediates are
processed more quickly into the final end product enkephalins; and 4) mutant PEs are ultimately more completely cleaved into enkephalins than native PE.

Several studies have demonstrated that adrenal medullary cell transplantation into rat spinal cord can be used as a method to produce and release opioid peptides for the treatment of chronic intractable pain (40–42). More recently, researchers have attempted to use cell therapy with genetically engineered cell lines to produce bioactive opioids to treat pain (43, 44). Implantation of genetically modified AtT-20 cells expressing peptide units per molecule of precursor. The mutant PEs described in this report may represent the first step in this direction.

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REFERENCES
1. Mains, R., Dickerson, I. M., May, V., Staffers, D. A., Perkins, S. N., Ouafik, L., Husten, E. J., and Ripper, B. A. (1990) Front. Neuroendocrinol. 11, 52–89
2. Rouillé, Y., Duguay, S., Lund, K., Furuta, M., Gong, Q., Lipkind, G., Oliva, A. A., Chan, S. J., and Steiner, D. (1995) Front. Neuroendocrinol. 16, 1–40
3. Brakch, N., Rholam, M., Boussetta, H., and Cohen, P. (1993) Biochemistry 32, 4925–4930
4. Seidah, N. G., Gaspar, L., Marcinkiewicz, M., Mbikay, M., and Chretien, M. (1990) DNA Cell Biol. 9, 415–424
5. Seidah, N. G., Marcinkiewicz, M., Bonnennet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M., and Chretien, M. (1991) Mol. Endocrinol. 5, 111–122
6. Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J., and Steiner, D. F. (1990) Proc. Natl. Acad. Sci. U.S.A. 88, 340–344
7. Smeekens, S. P., and Steiner, D. F. (1990) J. Biol. Chem. 265, 2997–3000
8. Mathis, J. P., and Lindberg, I. (1992) Endocrinology 131, 2287–2296
9. Udenfriend, S., and Kilpatrick, D. L. (1983) Arch. Biochem. Biophys. 221, 309–323
10. Wilson, S. P. (1991) J. Neurochem. 57, 876–881
11. Giles, N., Keutmann, H. T., and Mains, R. E. (1991) Mol. Endocrinol. 5, 404–413
12. Dickerson, I. M., Dixon, J. E., and Mains, R. E. (1990) J. Biol. Chem. 265, 2462–2469
13. Docherty, K., Rhodes, C. J., Taylor, N. A., Sheenan, K. I. J., and Hutton, J. C. (1989) J. Biol. Chem. 264, 18335–18339
14. Stoller, T. J., and Shields, D. (1989) J. Biol. Chem. 264, 6922–6928
15. Thorne, B. A., Caton, L. W., and Thomas, G. (1989) J. Biol. Chem. 264, 3545–3552
16. Jung, L., Kreiner, T., and Scheller, R. H. (1993) J. Cell Biol. 121, 11–21
17. Duguay, S. J., Lai-Zhang, J., and Steiner, D. F. (1995) J. Biol. Chem. 270, 17566–17574
18. Zhou, A., Bloomquist, B. T., and Mains, R. E. (1993) J. Biol. Chem. 268, 1763–1769
19. Rhodes, C. J., Lincoln, B., Shoelson, S. E. (1992) J. Biol. Chem. 267, 22719–22726
20. Yoshikawa, K., Williams, C., and Sabel, S. L. (1984) J. Biol. Chem. 259, 14301–14308
21. Lindberg, I., Shaw, E., Finley, J., Levine, D., and Deininger, P. (1991) Endocrinology 128, 1849–1856
22. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 488–492
23. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
24. Lindberg, I., and Zhou, Y. (1995) Methods Neurosci. 23, 94–108
25. Johanning, K., Mathis, J. P., and Lindberg, I. (1996) J. Neurochem. 66, 898–907
26. Lindberg, I., and Yang, H. Y. T. (1984) Brain Res. 299, 73–78
27. Lindberg, I. (1994) Mol. Cell. Neurosci. 5, 263–268
28. Mascetti, I., Giorgi, O., Schwartz, J. P., and Costa, E. (1994) Eur. J. Pharmacol. 266, 427–430
29. Lindberg, I. (1986) J. Biol. Chem. 261, 16137–16132
30. Giraud, P., Eiden, L. E., Audiger, Y., Gillioz, C., Bourdourque, F., Eskay, R., and Oliver, C. (1981) Neuropeptides 1, 257–252
31. Rostovtsev, A. P., and Wilson, S. P. (1994) Mol. Cell. Endocrinol. 101, 267–285
32. Wilson, S., Liu, F., and Housley, P. R. (1995) Soc. Neurosci. Abstr. 21, 1847
33. Zhou, A., and Mains, R. E. (1994) J. Biol. Chem. 269, 17440–17447
34. Christie, D. L., Batchelor, D. C., and Palmer, D. J. (1991) J. Biol. Chem. 266, 15679–15683
35. Vindrola, O., and Lindberg, I. (1992) Mol. Endocrinol. 6, 1088–1094
36. Milgram, S. L., and Mains, R. E. (1994) J. Cell Sci. 107, 737–745
37. Zhou, Y., and Lindberg, I. (1994) J. Biol. Chem. 269, 18408–18413
38. Day, N. C., Lin, Ueda, Y., Meador-Woodruff, J. H., and Akil, H. (1993) Neuropeptides 24, 253–262
39. Schiller, M. R., Mende-Mueller, L., Moran, K., Meng, M., Miller, K. W., and Hook, V. Y. H. (1995) Biochemistry 34, 7988–7995
40. Sagen, J., Pappas, G. D., and Pollard, H. B. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 7522–7526
41. Sagen, J., and Perlow, M. J. (1986) Brain Res. 384, 189–194
42. Hama, A. T., and Sagen, J. (1994) Brain Res. 651, 183–193
43. Takahashi, K., Fujita, T., and Takeuchi, T. (1995) Peptides 16, 92–93
44. Saitoh, Y., Arita, N., Ohnishi, T., and Hayakawa, T. (1995) Cell Transplant. 4, Suppl. 1, s13–s17
45. Wu, H. H., Wilcox, G. L., and McLean, S. C. (1994) J. Neurosci. 14, 4806–4814