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Intragranular Prolactin Phosphorylation and Kallikrein Cleavage Are Regulated by Zinc and Other Divalent Cations

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Rat prolactin (PRL) secretory granules contain enzymes for proteolytic cleavage and serial phosphorylation, but hormone cleavage products and phosphorylated PRL are not detected until just prior to exocytosis. Similarly, although PRL is stored in granules, in part, as high-mol-wt oligomers, PRL is primarily monomeric in the circulation. PRL secretory granules contain zinc, calcium, and magnesium, which inhibit depolymerization and dissolution of granules. Divalent cations also protect cysteine free thiol residues in the carboxy-terminal region of the intragranular hormone. The present studies examined the effect of removal and replacement of divalent cations on kallikrein cleavage and phosphorylation of secretory granule PRL.

Kallikrein cleavage was assessed utilizing two experimental protocols. First, granules were treated with or without 3 m*M* EDTA, free hormone thiols were alkylated, the PRL was cleaved by kallikrein, and the small kallikrein-cleavage peptides were assessed by reverse-phase HPLC. No differences in hormone cleavage owing to removal of divalent cations were observed at this concentration of EDTA. Second, divalent cations in granules were reduced/removed by 10 m*M* EDTA/3 m*M* *o*-phenanthroline (OP), followed by addition of either 5 m*M* zinc, magnesium, calcium, or additional EDTA. Kallikrein cleavage was then initiated. In this instance, the extent of proteolysis was analyzed by two-dimensional polyacrylamide gel electrophoresis (PAGE) of the larger remnant PRL pieces. After treatment with 10 m*M* EDTA/3 m*M* OP, results indicated that cleavage between R174 and R175 (site 1) was unaffected by added cations or additional EDTA. Recovery of site 2 cleaved PRL (L1-K185) and site 3 cleaved PRL (L1-R188) was ~40% reduced by zinc, but unaffected by calcium or magnesium. Additional EDTA resulted in increased recovery of site 2 cleaved PRL, but no change in site 3 recovery, suggesting the presence of tightly bound intragranular zinc around site 2, even after the initial EDTA/OP treatment.

Phosphorylation of PRL at S177 was studied using the same protocols. Phosphorylation was increased by added EDTA, even at 3 m*M*, and decreased by divalent cations, with no marked specificity for zinc observed. An additional experiment studied phosphorylation without exposure to kallikrein. Comparisons between the plus and minus kallikrein experiments showed kallikrein to have no apparent preference for unmodified or phosphorylated PRL.

From the kallikrein cleavage and phosphorylation studies and modeling of PRL, we suggest D181 as a likely site for intragranular zinc coordination. When C189 and C197 are present as free thiols in intragranular PRL, these may also contribute to binding. Zinc coordination in this region of the molecule apparently regulates proteolytic processing by kallikrein, as well as contributing to the stability of the hormone storage forms.

Key Words: Intragranular prolactin; divalent cation; kallikrein; phosphorylation; hormone posttranslational processing.

Introduction

Posttranslational processing of prolactin (PRL) is known to occur within secretory granules just prior to or during exocytosis (Ho et al., 1991; Anthony et al., 1993; Walker, 1994 [review]). The mechanisms involved in storing PRL in an osmotically inert state in granules (Greenan et al., 1990), maintenance of the intragranular milieu, and the triggering steps that allow hormone modifications and release, however, are not known in detail. The present studies investigated the potential role of zinc in rat (r) PRL storage and posttranslational processing. Zinc is present in millimolar concentrations in granules (Greenan et al., 1990), and it also inhibits PRL release in vitro and in vivo (reviewed in Lorenson et al., 1996). The mechanisms

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involved in zinc inhibition of release are not thoroughly understood, although at the granule level, it inhibits the conversion of intermolecularly associated hormone forms to releasable PRL (Lorenson et al., 1983). It may help stablize these oligomeric forms by protection of free cysteine thiol residues detected in the carboxy-terminal region of granule PRL (Lorenson et al., 1996). In addition, zinc deficiencies are associated with hyperprolactinemia in humans (Prasad, 1985).

The carboxy-terminal region of rPRL is the major site both of phosphorylation and kallikrein cleavage (Fig. 1). Phosphorylation in this region occurs at S177 (Wang et al., 1996), whereas kallikrein cleavage occurs among R174-175 (site 1), K185-F186 (site 2), and/or R188-C189 (site 3) (Fig. 1) (Powers and Hatala, 1990; Ho et al., 1991; Anthony et al., 1993). The functions of phosphorylated PRL and kallikrein-cleaved PRL, or the kallikrein-produced PRL peptides, however, have not been fully elucidated. One function of phosphorylated rPRL is to antagonize the proliferative effects of nonphosphorylated hormone in GH_3 (Krown et al., 1992) and Nb_2 lymphoma cells (Wang and Walker, 1993). Further, in normal pituitary cells, phosphorylated rPRL is responsible for autoregulation of PRL secretion (Ho et al., 1989), and during the estrous cycle, reproducible changes occur in released hormone phosphorylation states (Ho et al., 1993). No biological activities of the products of kallikrein have been demonstrated, but pituitary kallikrein is induced by estrogen (Clements et al., 1986; Hatala and Powers, 1987; Chao et al., 1987). Thus, physiological relevance is also suggested for kallikrein-cleaved PRL. Interestingly, zinc concentrations in the hypothalamus are also under estrous cycle regulation, and sex differences are seen in the pituitary (Merriam et al., 1979).

Within secretory granules, PRL concentrations are on the order of 50 m*M* (Lorenson et al., 1984). This high concentration is maintained, in part, by oligomerization of the hormone (Lorenson et al., 1984; Lorenson and Jacobs, 1987; Greenan et al., 1990), which most likely involves a combination of intermolecular disulfide bridges and intermolecular noncovalent bonds (reviewed in Lorenson et al., 1996). In contrast to monomeric PRL, intragranular rPRL has some cysteine free thiol residues in the carboxy-terminal region (Lorenson et al., 1996); these are protected by divalent cations (Lorenson et al., 1996). On removal of the coordinating cation, these thiols could become available for thiol:disulfide interchange reactions. Such reactions may result in the formation of intramolecularly disulfide-bonded monomers, increased intragranular osmotic pressure, and secretion (Lorenson and Jacobs, 1987). We know that enzymes required for serial phosphorylation (Greenan et al., 1989) and proteolytic cleavage (Powers and Hatala, 1990; Ho et al., 1991) of rPRL are present in mature secretory granules, and that intragranular packaging of rPRL protects the protein from phosphorylation (Greenan et al., 1989) and from carboxy-terminal cleavage by kallikrein

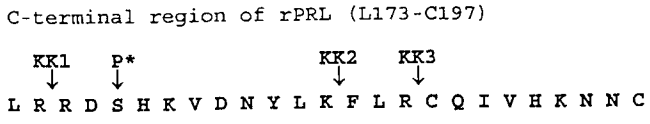

Fig. 1. Sequence of the carboxy-terminus of rPRL showing the sites of kallikrein cleavage (KK1, KK2, KK3) (Powers and Hatala, 1990) and the phosphorylation site (P*) (Wang et al., 1996).

(Powers and Hatala, 1990; Ho et al., 1991). In the present studies, we asked whether divalent cations, and specifically zinc, prevented these modifications. Utilizing data obtained showing zinc protection primarily in the region around amino acids 185 and 186, and a theoretical model for PRL based in part on the structure of GH (Abdel-Meguid et al., 1987), we suggest intragranular zinc coordination may include D181 and the cysteine thiols C189 and C197 if present.

Results

Divalent Cation Effects on Kallikrein Cleavage of Intragranular PRL

The influence of zinc, calcium, magnesium, and additional EDTA on in vitro kallikrein activity was tested before the effects of divalent cations on kallikrein cleavage of PRL were investigated. This enzyme is normally assayed in 10 m*M* EDTA/3 m*M* *o*-phenanthroline (OP) (Powers and Nasjletti, 1983), so this control was duplicating the conditions of granule digestion. Using the artificial substrate, D-Val-Leu-Arg-*p*-nitroaniline, we found all divalent cations (chloride salts) and additional EDTA inhibited activity (Fig. 2). Importantly, however, no significant difference was observed among inhibition by zinc, magnesium, or calcium. To ensure that the presence of divalent cations would not result in kallikrein being rate limiting when incubated with granules, the exogenous kallikrein concentration was increased in subsequent experiments.

When granules were exposed to 3 m*M* EDTA and free thiols were alkylated with 4-vinyl pyridine prior to kallikrein treatment, no differences owing to EDTA were observed in the total yield or relative yields of the small cleavage products on reverse-phase HPLC (Fig. 3). In contrast, dramatic differences were observed in two-dimensional gels when divalent cations were first extracted from granules with a higher concentration of EDTA (10 m*M* EDTA) plus 3 m*M* OP, followed by readdition of divalent cations (5 m*M*) or additional EDTA (to a final 15 m*M*) and kallikrein cleavage. Representative gels are shown in Fig. 4, and the average results of three experiments (24 gels) are presented in Fig. 5. The data for Fig. 5 were normalized to the percent cleavage in the control (43.0, 11.1, and 6.0% for sites 1, 2, and 3, respectively), because the extent of hydrolysis at each site was not equivalent. This allowed better graphical representation of the impor-

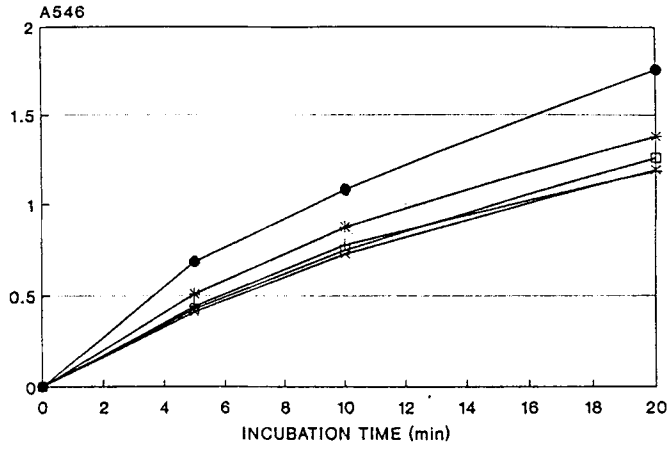

Fig. 2. Influence of divalent cations on kallikrein cleavage of D-Val-Leu-Arg-*p*-nitroaniline. Kallikrein (500 ng) was incubated with 400 μ*M* D-Val-Leu-Arg-*p*-nitroaniline as described in Methods without (●) or with additional 5 m*M* EDTA (x), 5 m*M* $MgCl_2$ (+), $CaCl_2$ (*), or $ZnCl_2$ (□). Production of *p*-nitro-aniline was monitored at 546 nm. Additions different from "without" sample (●). $p < 0.01$. Additions not significantly different from each other.

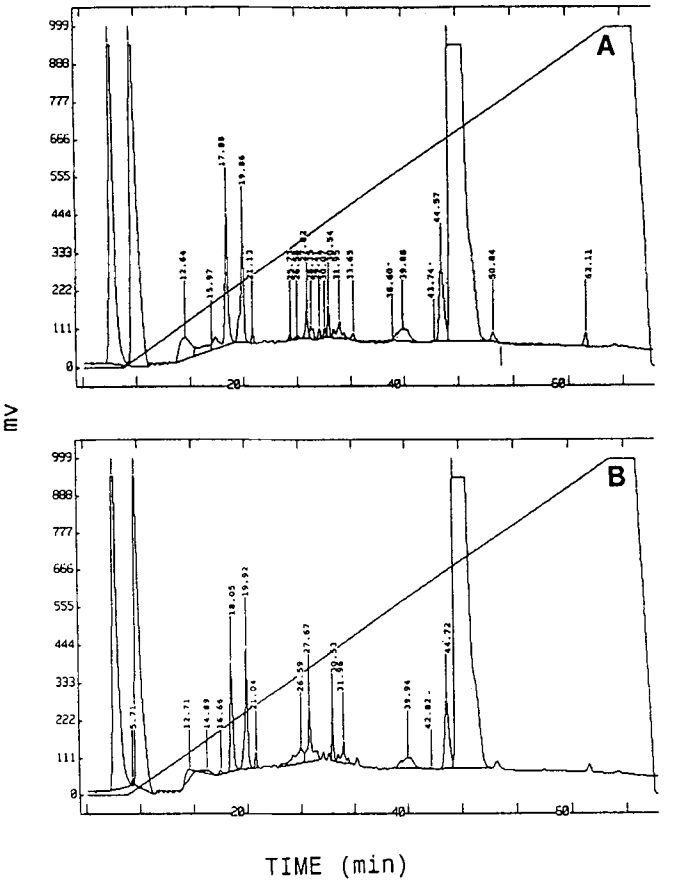

Fig. 3. HPLC elution profiles of kallikrein peptides produced with or without pretreatment of the granules with 3 m*M* EDTA in the presence of 4-vinyl pyridine. Treatment of granules with EDTA and alkylation of thiols are as described in Methods. Equal aliquots were loaded on the column (1 x 150 mm 5 μ 300A Vydac C18 column). Separation was achieved with a 60-min elution gradient from 0–60% acetonitrile. Panel **A** shows the results from granules not pretreated with EDTA and panel **B** those from granules exposed to EDTA. mv, millivolts.

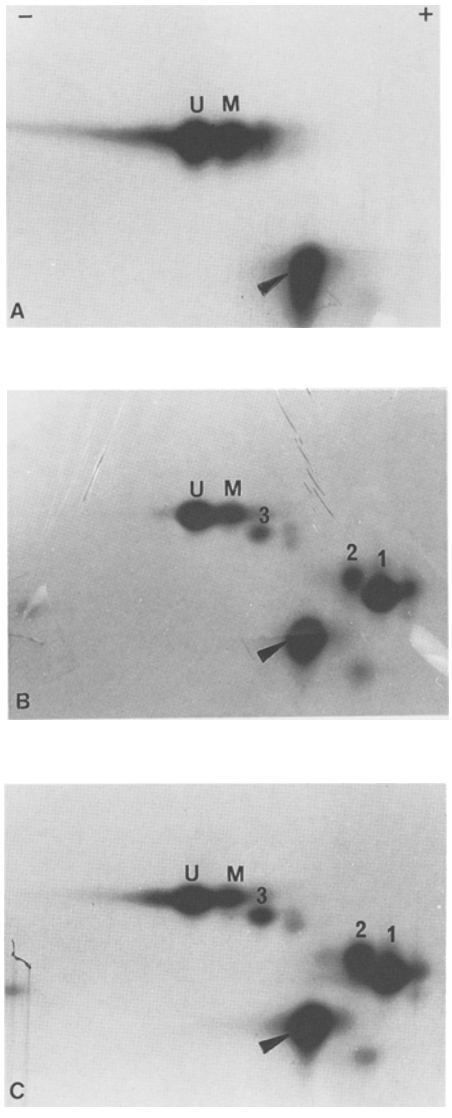

Fig. 4. Examples of two-dimensional PAGE of posttranslational modifications. Isolated granules were extracted with 10 m*M* EDTA/3 m*M* OP. No further additions were made to the granules shown in panel **A** before incubation at 37°C for 4 h. Granules shown in panels **B** and **C** were subsequently incubated with kallikrein (in the presence of 10 m*M* β-ME and Triton-X100) after the addition of 5 m*M* zinc (B) or additional 5 m*M* EDTA (C). U, unmodified PRL; M, monophosphorylated PRL; 1, 2, 3, products remaining after site 1, 2, and 3 cleavage by kallikrein, respectively. Arrow denotes mol wt and pI marker.

tant changes resulting from cation addition. The data first indicate that additional EDTA stimulated cleavage at site 2 by 40% without affecting sites 1 and 3. Thus more complete removal of divalent cations from site 2 allowed more cleavage to take place. Second, although addition of zinc had no effect on site 1, it markedly inhibited hydrolysis at sites 2 and 3, reducing values compared to control by 42 and 39%, respectively. If compared to conditions with additional EDTA, the effect of added zinc was even more marked at site 2. Third, inhibition by magnesium and calcium was less than 20% at any site; in fact, at site 2, the

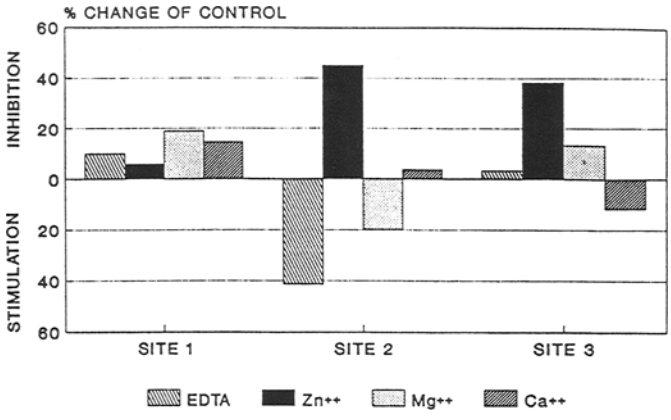

Fig. 5. Influence of divalent cations on kallikrein cleavage of intragranular rPRL. Isolated granules were extracted with 10 m*M* EDTA/3 m*M* OP, before addition of either 5 m*M* $ZnCl_2$, $MgCl_2$, $CaCl_2$, or additional EDTA and kallikrein as described in Methods. Cleavage at the three known rPRL sites (Ho et al., 1991; Powers and Hatala, 1990) was assessed by two-dimensional PAGE, silver staining, and densitometry as described (Ho et al., 1991). Depicted in the figure are the average results of three separate experiments. These data were obtained from 24 gels similar to those shown in Fig. 4. The effects of zinc at sites 2 and 3 and the effect of additional EDTA at site 2 are significantly different from the control $p < 0.01$.

presence of magnesium appeared to increase cleavage. From these data, then, it appears that (1) zinc coordinates with PRL in such a way as to block kallikrein cleavage of sites 2 and 3, but not site 1, and (2) protection appears greatest at site 2.

Divalent Cation Influence on Phosphorylation of Intragranular rPRL

The influence of intragranular divalent cations on rPRL phosphorylation was studied first by sequence analysis of a small carboxy-terminal region, kallikrein-cleaved peptide. Granules were treated with 4-vinyl pyridine in the absence or presence of 3 m*M* EDTA, followed by kallikrein treatment, isolation of the small peptide products by reverse-phase HPLC, and sequencing. The relative degree of phosphorylation was assessed by determining the amount of S177, phosphorylation being indicated by a loss of serine recovery. As shown in Table 1, the amount of serine was low (0.4 pmol) when granules were incubated with EDTA compared to when granules were incubated without EDTA (2.1 pmol). It should be pointed out that differences in total peptide recovery were not a function of EDTA, but were due to sample loading on the Porton disks during analysis. Different loading was to ensure that a measurable amount of serine was recovered in the "plus EDTA" sample. When the amount of total peptide in each analysis was taken into account, the marked difference in serine recovery was even more evident. If recoveries were approximated using the average picomolar yield of each amino acid through the

Table 1
Influence of EDTA on Phosphorylation: Recovery of Serine 177 During Sequence Analysis of a Kallikrein-Cleaved Peptide

Residue			Recovered amino acid, picomoles recovered	
Cycle	Residue	AA	–EDTA	+EDTA
1	175	R	13.9	62.7
2	176	D	15.5	35.7
3	177	S	2.1	0.4
4	178	H	6.5	10.2
5	179	K	8.9	15.5
Mean without serine			11.2	31.0

Secretory granule rPRL (40 μg) was incubated with 0.2 m*M* 4-vinyl pyridine in the absence or presence of 3 m*M* EDTA followed by kallikrein cleavage and isolation of the carboxy-terminal peptides. Sequencing of the peptide isolated by reverse-phase HPLC at 45.74 min through nine cycles showed RDSHKVDNY. Only the first five cycles of sequencing are shown for simplicity. Loss of serine is indicative of phosphorylation.

five cycles shown (except serine), then an average recovery of 31.0 pmol with EDTA compared to 11.2 pmol without EDTA was obtained. Thus, the serine lost with EDTA (phosphorylated serine) (31-0.4/31) was greater than the serine lost (11.2-2.1/11.2) in the absence of EDTA. These data provide strong evidence that under these conditions, much more S177 was phosphorylated in the presence of EDTA.

The influence of individual divalent cations was also tested. These experiments, like those for kallikrein, first required removal of intragranular divalent cations with EDTA and OP, and then their individual replacement. Phosphorylation was monitored by densitometric analysis of two-dimensional gels as previously described (Ho et al., 1993). As shown in Table 2, each divalent cation inhibited phosphorylation, with no specificity for zinc being apparent.

To investigate the possibility that phosphorylation influenced kallikrein cleavage and hence the small peptide results, we conducted a similar experiment in which granular PRL was subsequently exposed to kallikrein under the usual cleavage conditions (i.e., in the presence of 0.25% Triton X100 and 10 m*M* β-mercaptoethanol (β-ME). Because of the low yield of one large remnant cleavage product, it is not possible to quantify accurately and separately the phosphorylated and nonphosphorylated versions of this product on silver-stained gels. We therefore have tabulated only the uncleaved PRL in terms of nonphosphorylated and monophosphorylated forms. By analysis of what is not cleaved, there is no evidence that kallikrein preferentially cleaves either unmodified (U) or monophosphorylated PRL (M). The M/U ratio was the same for the control with or without kallikrein cleavage.

Table 2
Effect of Cations on PRL Phosphorylation

	Isoform 1	U	M	D	Ratio M/U
	(Percent of Total PRL)				
No kallikrein cleavage					
Control (10 m*M* EDTA/3 m*M* OP)	4	58	37	0	0.63
Zn^{2+} (5 m*M*)	7	61	28	3	0.45
Mg^{2+} (5 m*M*)	9	64	27	0	0.42
Ca^{2+} (5 m*M*)	8	63	26	2	0.41
With kallikrein cleavage*					
Control (10 m*M* EDTA/3 m*M* OP)	0	23	15	1	0.65

Ions were added to granules suspended in 10 m*M* EDTA/3 m*M* OP. The data are expressed as a percentage of the total PRL on the gel. This form of expression corrects for any gel-loading errors. Values have been rounded to the closest whole number. Isoform 1 is an intragranular posttranslational modification of unknown nature; U, represents unmodified PRL; M, represents monophosphorylation of U at serine 177; D, represents the usually minor diphosphorylated variant. Note the decreased percentage of monophosphorylated PRL found when Zn^{2+}, Mg^{2+}, or Ca^{2+} ions were readded to the granules. The ratio of monophosphorylated to unmodified PRL dropped from 0.63 to an average of 0.43. These results are representative of four similar separate experiments. *Only uncleaved PRL represented.

Discussion

Prolactin is stored in secretory granules in very high concentration (Lorenson et al., 1984; Lorenson and Jacobs, 1987). This high concentration is osmotically inert owing in large part to oligomerization of the molecules (Greenan et al., 1990). Recently, we found evidence that some carboxy-terminal cysteines exist as free thiols in granules and that these are protected by divalent cations (Lorenson et al., 1996). It is speculated that protection of these thiols by divalent cations may stabilize intragranular hormone forms. Conversely, removal of divalent cations may expose hormone thiols, resulting in the initiation of thiol:disulfide interchange reactions leading to hormone monomerization and exocytosis (Lorenson et al., 1996). We also know that PRL stored in secretory granules is unmodified (Greenan et al., 1989), meaning that the hormone is primarily nonphosphorylated and PRL cleavage products of kallikrein are not present. This is despite the fact that in granules, enzymes responsible for phosphorylation of PRL (Greenan et al., 1989) and specific carboxy-terminal cleavage (Powers and Hatala, 1990; Ho et al., 1991) are present. These posttranslational modifications therefore are kept in check inside the granule until just before exocytosis. In the present study, we investigated the effect of intragranular divalent cations on these posttranslational modifications. Despite the fact that PRL has yet to be crystallized and the structure determined, the data obtained combined with information on GH structure (Abdel-Meguid et al., 1987) suggested a likely binding site of zinc in intragranular PRL. All of the more traditional approaches used to study metal–protein interactions cannot be applied to granule storage forms, since they all require the prolactin, at least initially, to be in solution.

The present results indicate that zinc binding to intragranular hormone is relevant to kallikrein cleavage. The sites of cleavage by this enzyme in the carboxy-terminal region of the hormone have been carefully analyzed by Powers and Hatala (1990). This enzyme is normally present in PRL granules, a proportion of it is in the active form (Ho et al., 1991; Wang et al., 1996), and PRL cleavage products produced by catalysis are secreted from cells (Meriam et al., 1979; Oetting and Walker, 1985; Oetting et al., 1989; Powers and Hatala, 1990). Whether direct or indirect, zinc protection of cleavage sites may be important functionally. Additionally, pituitary concentrations of both the kallikrein and zinc are under estrogen (E_2) control (Hatala and Powers, 1987; Chao et al., 1987; Ho et al., 1993). Further, differences in susceptibility to hydrolysis have been found when granules from E_2-treated or untreated animals were incubated with exogenous kallikrein (Greenan et al., 1989). Thus, even though we added more kallikrein to the granules to conduct the experiments, we were nevertheless monitoring a natural posttranslational modification.

Assays for glandular kallikrein activity are normally conducted in the presence of chelating agents, because divalent cations are known to inhibit activity (Fiedler, 1979). We confirmed and extended this finding in the present studies where we monitored enzyme activity using the artificial substrate D-Val-Leu-Arg-*p*-nitroaniline. We found that 5 m*M* levels of all divalent cations tested (calcium, magnesium, and zinc) inhibited enzyme activity by

~25% (assay buffer contained 10 m*M* EDTA/3 m*M* OP) (Fig. 2). Additional kallikrein was utilized in granule incubations to minimize the possible direct divalent cation inhibition on the enzyme. This effectively negated the inhibition on the enzyme *per se*, since neither zinc nor other divalent cations inhibited cleavage at site 1 compared to controls (Fig. 5).

Clear evidence of zinc protection against kallikrein cleavage at sites 2 and 3 is seen in Fig. 5. This cation was much more effective than either magnesium or calcium, two divalent cations also present in granules in millimolar concentrations (Greenan et al., 1990). It should again be pointed out that no effect of zinc or other divalent cations on site 1 was observed. Since site 1 is the major cleavage site and is unaffected, this is a good indicator that the affect of zinc is not on the catalytic activity of the enzyme, but rather is on the PRL. Since 3 m*M* EDTA prior to reduction and cleavage had no effect on either the total yield or proportion of cleavage at each site (Fig. 3), it is likely that the intragranular divalent cation relevant to kallikrein cleavage is very tightly bound and not removed by this concentration. This is supported by the fact that cleavage in 15 m*M* EDTA/3 m*M* OP specifically stimulated hydrolysis at site 2 compared to cleavage in controls (10 m*M* EDTA/3 m*M* OP) (Fig. 5).

Phosphorylation of serine 177 in intragranular PRL is blunted by divalent cations (Fig. 4, Tables 1 and 2) and, unlike kallikrein cleavage, is increased by 3 m*M* EDTA. Also, no significant distinction among divalent cations was observed at concentrations we have determined show differences in kallikrein cleavage. It therefore appears that the mechanism for divalent cation inhibition of phosphorylation is distinct from that which inhibits kallikrein cleavage. Since the intragranular kinase has yet to be isolated, we cannot yet determine the relative contributions of direct cation inhibition of the enzyme and cation protection of the rPRL phosphorylation site.

Regardless of the mechanism, it is clear that divalent cations protect intragranular PRL from phosphorylation. Results that corroborate divalent cation protection of phosphorylation are the findings that (1) the depletion/transformation phenomenon in pituitaries of lactating rats correlates with PRL phosphorylation (Montiel et al., 1993), and (2), this loss of detectability prior to hormone release (depletion) is strongly influenced by zinc (the only cation tested) (Martinez-Escalera et al., 1986).

When PRL is modeled according to the known structure of GH (Abdel-Meguid et al., 1987), all three kallikrein sites and the phosphorylation site are in helix 4 (Fig. 6). The helix terminates at I191, where the carboxy-terminal disulfide loop begins. This disulfide loop, in which C197 binds to C189, exists in monomer PRL. In intragranular PRL, C197 and C189 may be involved in intermolecular disulfide bonds; some may also exist as thiols, possibly being coordinated to divalent cations (e.g., zinc) (Lorenson et al., 1996). Assuming maintenance of the helix in intragranular PRL, zinc-binding amino acids that are in close proximity to kallikrein cleavage site 2 are D181, C189, and C197 (if the latter two are present as free thiols and the last is poised to form the C189-C197 disulfide when oxidized). Zinc coordination among these amino acids would also likely overlap site 3, thus affording it some protection. D181 in PRL is the equivalent site to E174 in GH, which has been shown to be an important site of zinc binding in the latter hormone (Cunningham et al., 1991).

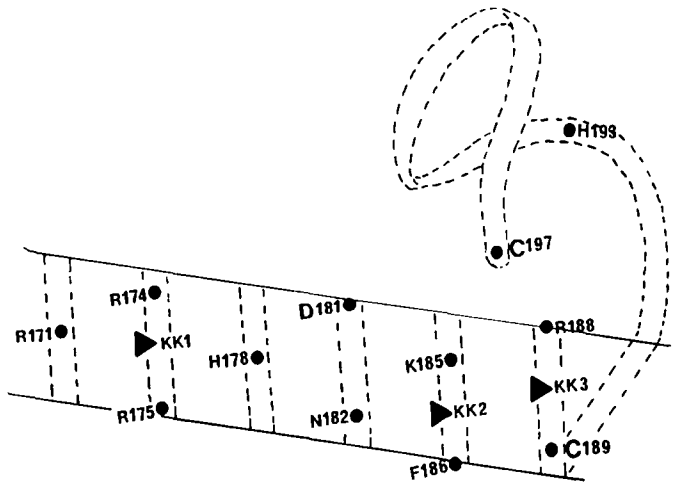

Fig. 6. Schematic representation of the carboxy-terminal region of rPRL (R171-C197). One face of a portion of Helix 4 and the carboxy-terminal tail region of rPRL are illustrated. Amino acids in the helix are assumed to take a 100° arc. The cleavage sites for kallikrein (KK1, KK2, and KK3) are indicated by triangular arrowheads. The disulfide tail is illustrated above the helix so as not to obscure the kallikrein cleavage sites and to illustrate the potential freedom of C197 when it is not disulfide-bonded to C189.

Figure 6 also shows a potential zinc-binding site very close to kallikrein site 1, H178, but coordination with zinc here seems less likely, since we found no evidence of zinc binding to H178 from the kallikrein cleavage studies (i.e., no inhibition of site 1 cleavage). Mutational studies performed on the equivalent residue in GH support this view: mutation to alanine had no effect on zinc binding (Cunningham et al., 1991), although the limitations on this analogy are that the GH was monomeric and not intragranular. The phosphorylation site is on the back side of the helix as it is displayed in the figure and, hence, may be away from the region of zinc coordination around D181. This may explain the different divalent cation dependency of phosphorylation and kallikrein cleavage. The phosphorylation site is also flanked by D176, which could be involved in divalent cation/phosphorylation interrelationships. Depending on the freedom of the carboxy-terminal tail, another potential coordination site is H193. The present results give us no insight regarding these amino acids.

Species differences in kallikrein cleavage and phosphorylation of intragranular PRLs may be related to hormone divalent cation-binding ability. The amino acid candidates for divalent cation binding in the carboxy-terminal region of PRLs are highly conserved (Luck et al., 1989). D181 and D176 (rPRL) occur in all PRLs, and H178 and H193 are only substituted in other species with other zinc-binding amino acids (S or Y), although these have lower affinity. Thus, a range of zinc-binding affinities could exist (Lorenson et al., 1996).

In summary, we have found evidence that divalent cations in general protect intragranular PRL from kallikrein cleavage and phosphorylation. This protection may be through binding to the hormone and/or, in the case of phosphorylation, by inhibition of enzyme activity. Specifically, zinc coordination to PRL in secretory granules protects two sites of kallikrein cleavage (sites 2 and 3), thereby promoting the production of the kallikrein site 1-cleaved products, a 21-kDa protein (Greenan et al., 1989; Ho et al., 1991; Anthony et al., 1993) and a 23 amino acid peptide (RDSHKVDNYLKFLRCQIVHKNNC). We suggest that the most likely binding site for zinc is D181. When C189 and C197 are present as free thiols in intragranular rPRL, these may also contribute to zinc coordination.

Materials and Methods

Materials

Divalent cations (chloride salts), EDTA, OP (a zinc chelator), sucrose, 4-vinyl pyridine, kallikrein substrates, Ellman's reagent (Ellman, 1959), and buffers were purchased from Sigma Chemical Co. (St. Louis, MO), whereas Centricon centrifugal concentrators were from Amicon Corp. (Beverly, MA). Mature female Sprague-Dawley-derived rats weighing 200–250 g were obtained from Bantin and Kingman Co. (Fremont, CA). Rat urinary glandular kallikrein was a generous gift of Dr. Julie Chao, Medical College of South Carolina (Charleston, SC). All other chemicals and materials were obtained at the highest available purity.

Methods

Isolation of PRL Secretory Granules

Pituitary subcellular fractionation was conducted as described by Zanini and Giannattasio (1973) with modifications as described previously (Ho et al., 1991). Briefly, pituitaries from 24 female rats (200–250 g) at random stages of the estrous cycle were homogenized in 0.32*M* sucrose, followed by removal of debris and nuclei by a low-speed centrifugation and pelleting of organelles by centrifugation for 30 min at 15,000*g*. Pellets were treated with 0.5 m*M* puromycin in sucrose containing 1*M* KCl/5 m*M* $MgCl_2$ to lyse most GH granules and to remove contaminating microsomes (by stripping off ribosomes and thereby increasing buoyancy). The puromycin-treated material was then loaded on discontinuous sucrose gradients and the granule fraction isolated as described (Zanini and Giannattasio, 1973). After washing the pellet in 0.32*M* sucrose and resedimenting at 30,000*g* for 60 min, granules (~200 μg) were suspended in 0.2 mL 0.32*M* sucrose and stored at −70°C. The amount of PRL/μL was estimated by reducing PAGE and comparison to standards. This granule suspension was relatively free of contaminants, as indicated by ultrastructural and biochemical criteria (Zanini and Giannattasio, 1973; Ho et al., 1991). Just prior to use in experiments, granules were sonicated for 10 s in the frozen state on ice at position 10 on a Kontes micro-ultrasonic cell disruptor. All procedures involving live animals were approved by the University of California at Riverside Committee on Laboratory Animal Care and were in compliance with NIH guidelines for animal care.

Effect of Divalent Cations and EDTA on Kallikrein Activity

The effects of divalent cations on kallikrein activity were studied using rat urinary glandular kallikrein which is identical to pituitary secretory granule kallikrein (Chao et al., 1987; Powers and Hatala, 1990; Ho et al., 1991). Kallikrein activity was determined with D-Val-Leu-Arg-*p*-nitroaniline (S-2266) as described by Powers and Nasjletti (1983). Briefly, 400 μ*M* peptide substrate was incubated with 500 ng kallikrein in 0.5 mL buffer containing 10 m*M* Tris-HCl (pH 8.5), 0.15*M* NaCl, 10 m*M* EDTA, and 3 m*M* OP without or with either 5 m*M* $MgCl_2$, $ZnCl_2$, $CaCl_2$, or additional EDTA. Samples were incubated at 37°C for 5–20 min, and then cleavage was stopped by heating at 100°C for 5 min. The reaction mixture was acidified with 0.2 mL 20% perchloric acid, placed on ice for a minimum of 10 min, and the protein precipitate removed by centrifugation at 10,000*g* for 5 min. The diazonium salt was formed by treatment of the supernate (0.5 mL) with 0.5 mL 0.2% sodium nitrite at 0–4°C for 10 min, removal of excess sodium nitrite with 0.5 mL 0.5% ammonium sulfamate, and addition of 0.5 mL 0.1% *N*-1-napthylethylenediamine-HCl in methanol. After incubation for 10 min at 22°C in the dark, the amount of the purple, azo dye formed was measured at 546 nm. Peptide hydrolysis was quantified by comparison with concentrations of standard *p*-nitroaniline diazotized under similar conditions, including added divalent cations and/or additional EDTA.

Kallikrein Cleavage of Secretory Granule PRL: Assessment by Reverse-Phase HPLC of Small Peptides Cleaved by Kallikrein

Forty microliters of granules (1 μg PRL/μL) were incubated without or with 3 m*M* EDTA under vacuum in 5 m*M* TrisHCl (pH 7.5)–0.2 m*M* 4-vinyl pyridine; 4-vinyl pyridine alkylated any free thiols as described (Wu et al., 1971; Lorenson et al., 1996). After 2 h at 24°C, samples were washed twice with 0.01*N* acetic acid by Centricon-10 filtration centrifugation, followed by lyophilization, solubilization in

10 m*M* Tris-HCl (pH 8.0)–10 m*M* β-ME–0.06% Triton X100, and incubation at 37°C for 4 h with kallikrein (1 μg) (Ho et al., 1991; Wang et al., 1996). Carboxy-terminal peptide fragments were then separated from the large 21 kDa cleavage fragment (and undigested PRL) by Centricon-10 filtration as previously described (Lorenson et al., 1996). Potential differences in kallikrein cleavage due to EDTA were assessed by comparing peptide patterns produced by reverse-phase HPLC (C18, 5 μ, 1 × 150 mm, 300A Vydac column). HPLC isolation of peptides was carried out at the University of California Riverside Biotechnology Instrumentation Facility.

Kallikrein Cleavage of Secretory Granule PRL: Assessment by Two-Dimensional PAGE of PRL and Its Large Kallikrein-Cleaved Products

Divalent cations were reduced/removed from isolated granules by suspension of granules in 5 m*M* Tris-HCl–0.15*M* NaCl buffer (pH 8.5) containing 10 m*M* EDTA, 3 m*M* OP for 10 min at room temperature. To granule aliquots (5 μg) was then added either $ZnCl_2$, $MgCl_2$, $CaCl_2$ (5 m*M* final), or additional EDTA (15 m*M* final), followed by incubation with kallikrein (50 ng) for 4 h at 37°C in the presence of 0.25% Triton X-100 and 10 m*M* β-ME. The large cleavage products from proteolysis at sites 1, 2, and 3 (L1-R174, L1-K185, and L1-R188, respectively) were quantitatively identified by two-dimensional PAGE and densitometry of silver-stained gels as previously described (e.g., Greenan et al., 1989; Ho et al., 1991; Ho et al., 1993). It should be noted that these kallikrein-cleaved products are the large protein fractions remaining, and not the smaller carboxy-terminal fragments isolated and sequenced as described above.

Phosphorylation of Secretory Granule PRL: Assessment of S177 Phosphorylation by Sequencing of Small Peptides Cleaved by Kallikrein

Kallikrein cleavage at site 1 (R174-R175) with or without cleavage at sites 2 and 3 results in peptide(s) containing the major phosphorylation site of rPRL, S177 (Wang et al., 1996), as illustrated in Fig. 1. The extent of phosphorylation can be assessed on sequencing of these peptides, since the change in charge owing to phosphorylation of serine results in its immediate elution off the sequencing column, with subsequent loss of serine recovery from that cycle (Glazer et al., 1975). Thus, to assess the effect of intragranular divalent cations on phosphorylation, granular PRL was first alkylated in the presence or absence of 3 m*M* EDTA and then treated with kallikrein as described above. The peptide fraction eluting at 45.74 min on reverse-phase HPLC, which contained R174-C197, was sequenced on a Porton disk by stepwise digestion and chromatography using Applied Biosytems 475A software for analysis. These analyses were carried out at the University of California Riverside Biotechnology Instrumentation Facility.

Phosphorylation of Secretory Granule PRL: Assessment by Two-Dimensional PAGE of Phosphorylated PRL

S177-phosphorylated isomers of intragranular rPRL and rPRL cleaved by kallikrein at sites 2 and 3 are more negatively charged than their nonphosphorylated forms and have been identified by two dimensional PAGE (Greenan et al., 1989; Ho et al., 1991, 1993). In the present study, we analyzed the effects of divalent cations on the presence of unmodified, non-kallikrein cleaved hormone (U) and its monophosphorylated isomer (M). The phosphorylated, kallikrein-cleaved products were not quantified owing to overlap of spots and/or low yields. Isolated granules were exposed to buffer containing 10 m*M* EDTA and 3 m*M* OP, followed by incubation with either 5 m*M* zinc, magnesium, calcium, or additional EDTA. Two sets of experiments were then carried out. In the first, granule incubation mixtures were then warmed for 4 h at 37°C followed by two-dimensional PAGE and silver staining. In the second, granules were incubated as above, but with the addition of kallikrein (50 ng for 4 h at 37°C), Triton X-100 (0.25%), or β-ME (10 m*M*).

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