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# Prolactin-Releasing Peptide and Its Homolog RFRP-1 Act in Hypothalamus but Not in Anterior Pituitary Gland to Stimulate Stress Hormone Secretion

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**The RF-amide peptides (RFRPs), including prolactin (PRL)-releasing peptide-31 (PrRP-31) and RFRP-1, have been reported to stimulate stress hormone secretion by either direct pituitary or indirect hypothalamic actions. We examined the possible direct effects of these peptides on PRL and adrenocorticotropin (adrenocorticotropic hormone [ACTH]) release from dispersed anterior pituitary cells in culture and on PRL and ACTH secretion following intracerebroventricular (icv) administration in vivo. Neither peptide significantly altered PRL or ACTH release from cultured pituitary cells (male rat donors). Central administration of 1.0 and 3.0 nmol of PrRP-31, but only the higher dose of RFRP-1, significantly elevated serum corticosterone levels in conscious male rats. The effect of PrRP-31 was not blocked by pretreatment (iv) with the corticotropin-releasing hormone (CRH) antagonist, α-helical CRH 9-41; however, pretreatment of the animals (iv) with an antiserum to CRH significantly lowered the hypothalamic-pituitary-adrenal axis response to central administration of PrRP-31. On the other hand, the release of PRL was significantly elevated by 3.0 nmol of RFRP-1, but not PrRP-31, in similarly treated, conscious male rats. Pretreatment with the catecholamine synthesis inhibitor, α-methyl-para-tyrosine, prevented the stimulation of PRL secretion observed following central administration of RFRP-1. RFRP-1 similarly did not alter PRL secretion in rats pretreated with the dopamine, $D_2$ receptor blocker, domperidone. These results suggest that the RF-amide peptides are not true neuroendocrine regulators of stress hormone secretion in the rat but, instead, act centrally to alter the release of neuroendocrine factors that do act in the pituitary gland to control PRL and ACTH release. In the case of RFRP-1, stimulation of PRL secretion is potentially owing to an action of the peptide to inhibit dopamine release into the median eminence. The corticosterone secretion observed following central administration of PrRP-31 does not appear, based on our current results, to be solely owing to an action of the peptide on CRH-producing neurons but, instead, may be a result of the ability of PrRP-31 to increase as well the exposure of the corticotrophs in vivo to other ACTH secretagogues, such as oxytocin or vasopressin.**

**Key Words:** Prolactin; adrenocorticotropin; neuroendocrine; dopamine; releasing factors.

## Introduction

In 1998, Hinuma et al. *(1)* reported the identification of two peptides in hypothalamic extracts that bound to the orphan receptor, UHR-1, and its human counterpart hGR3. Since UHR-1 was highly expressed in the anterior lobe of the pituitary gland, they hypothesized that these newly described peptides might act as neuroendocrine factors in the gland. Indeed, they reported that the peptides selectively, and in a concentration-related fashion, stimulated prolactin (PRL) release in vitro *(1)*. These two ligands were named PRL-releasing peptides (PrRPs), based on those initial in vitro studies, and specifically PrRP-31 and PrRP-20, based on their amino acid contents. They are posttranslational products of the same prohormone, PrRP-20 being the C-terminal 20 amino acids of PrRP-31 *(1)*. We subsequently reported that the PrRPs were weak PRL-releasing factors in cultured rat pituitary cells, and then only at very high concentrations in cultures of cells harvested from female rats *(2)*. These observations, coupled with the facts that only sparse PrRP-positive axon terminals or fibers could be visualized in the external layer of the median eminence *(3,4)* and that only very high doses of the peptide were capable of altering PRL release in vivo when administered intravenously *(5)*—if any effect actually could be observed *(6)*—cast doubt on the hypothesis that these peptides were phys-

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iologic regulators of adenohypophysial PRL release. However, PrRP-positive cells and PrRP receptors have been localized to central nervous system (CNS) sites recognized to be important in the control of autonomic nervous system (ANS) function and in the neuroendocrine response to stress *(5–8)*. Indeed, central, but not peripheral, administration of PrRP-20 and PrRP-31 in conscious male rats resulted in significant elevation in blood pressure and heart rate *(9)*, further suggesting a neuromodulatory role for the peptides in the control of ANS function.

While in vivo experiments on the pharmacologic effects of the PrRPs were being conducted in several laboratories *(2,5,6)*, the Hinuma group at Takaeda *(10)* reported the discovery in brain extracts of novel structural homologs of the PrRPs, the RF-amide peptides (RFRPs). The PrRPs and RFRPs belong to a family of peptides that share extensive homology and a common RF(arginine-phenylalanine)-amide C terminus. RFRP-immunoreactive fibers are also localized to CNS sites known to be important in the control of ANS function and the neuroendocrine response to stress *(10)*. Like PrRP, no significant accumulation of RFRP immunoreactivity was detected in the median eminence. This suggested to the peptides' discoverers that RFRP might act within the hypothalamus to control stress hormone release, and, indeed, they presented evidence for such a role, demonstrating the ability of centrally administered RFRP to result in elevated circulating PRL levels *(10)*.

We report here in vitro and in vivo studies designed to identify the sites of action of PrRP and RFRP to alter stress hormone secretion. While direct pituitary sites of action of the peptides to alter the secretion of PRL and adrenocorticotropic hormone (ACTH) release were not observed, our data suggest hypothalamic actions of the peptides to inhibit dopamine (in the case of RFRP) and stimulate the secretion of ACTH-releasing factors (in the case of PrRP) into the hypophysial portal circulation.

## Results

### *In Vitro Studies*

We have previously reported that PrRP failed to significantly alter PRL release from dispersed anterior pituitary cells harvested from male rat donors *(2)*, and in the present studies, once again PrRP failed to significantly alter basal PRL release (data not shown). Similarly, RFRP did not significantly alter PRL release in this cell model across the same concentration range (control: 72 ± 5 ng of PRL released in 1 h, $n = 12$; RFRP, all $n = 6$, 71 ± 6, 78 ± 8, 77 ± 7, 78 ± 7, and 75 ± 6 for 1000, 100, 10, 1, and 0.1 n*M*, respectively).

Neither PrRP nor RFRP significantly altered basal ACTH release from dispersed anterior pituitary cells (Fig. 1). Additionally, neither peptide interfered with or augmented the ACTH release observed in response to corticotropin-releasing hormone (CRH).

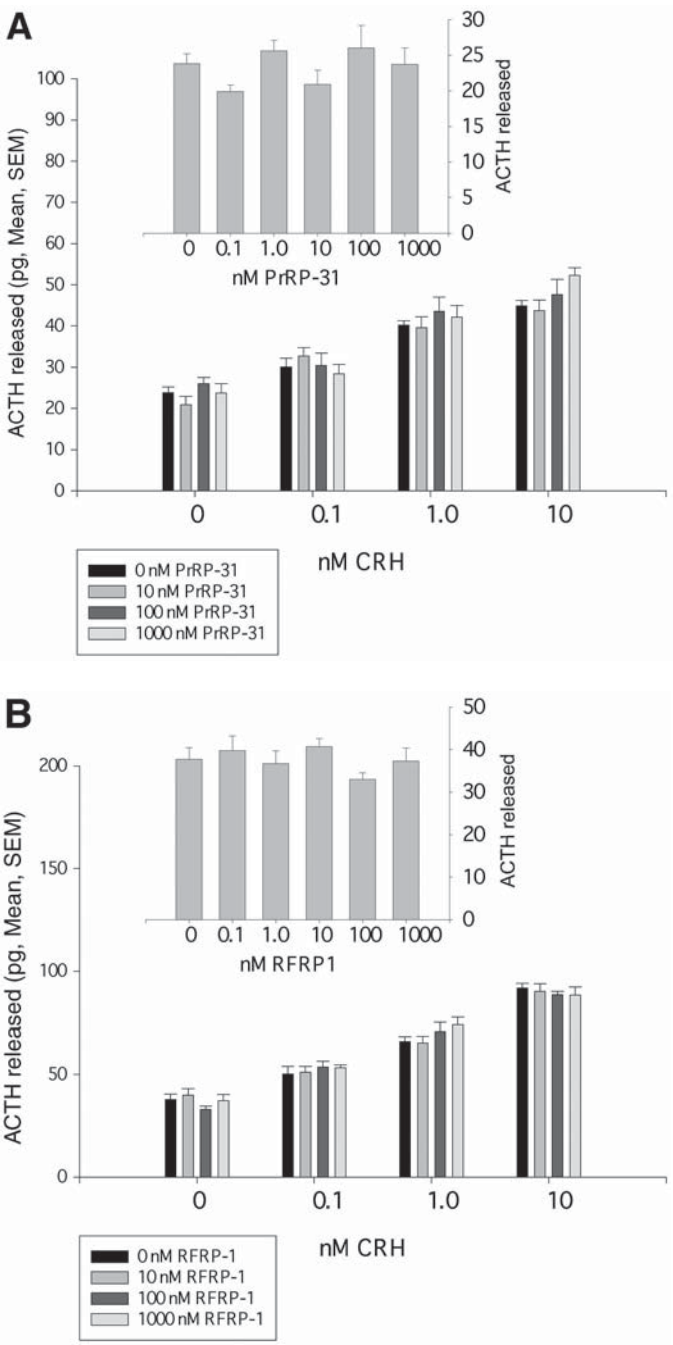


**Fig. 1.** (**A**) PrRP-31 and (**B**) RFRP-1 failed to significantly alter basal (inset) or CRH-stimulated adrenocorticotropin (ACTH) release from dispersed, anterior pituitary cells harvested from male rats.

### *In Vivo Studies*

Administration of 2-µL of saline vehicle into the lateral cerebroventricle of conscious male rats failed to significantly alter circulating levels of PRL (Fig. 2). When serum PRL levels were compared at each time point between treatment groups, both doses of RFRP (1.0 and 3.0 nmol) resulted in significant changes. An abrupt, fivefold increase in serum PRL levels was observed in the 3.0-nmol RFRP group 15

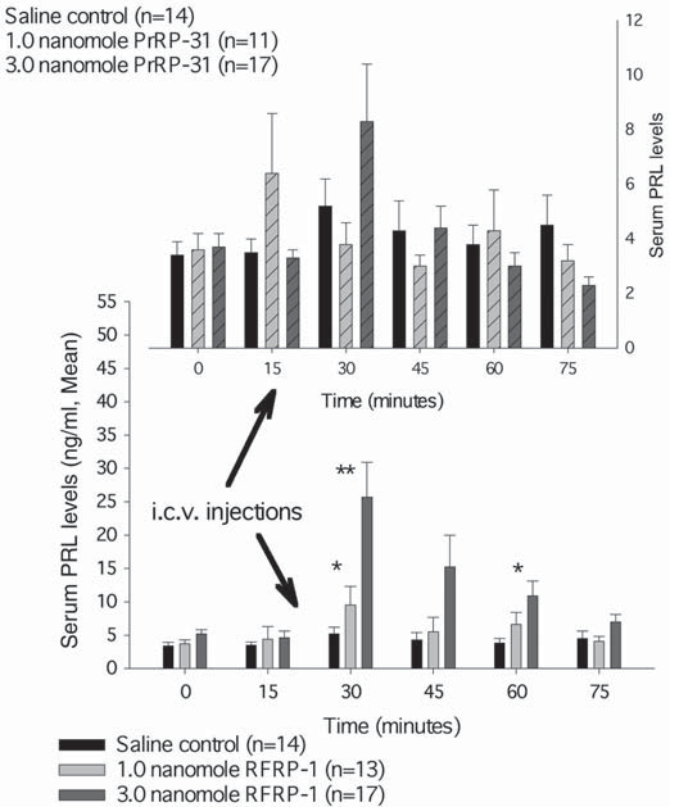


**Fig. 2.** Central administration of RFRP-1 significantly elevated serum PRL levels in conscious, unrestrained male rats. Similar injections of PrRP-31 were without significant effect (top). *$p < 0.05$, **$p < 0.01$ vs saline-injected controls at the indicated sample collection.

min after peptide administration ($p < 0.001$ vs PRL levels in control animals at that sampling). Serum PRL levels in these animals remained elevated for 30 more min, the effect being statistically significant ($p < 0.05$ compared to control) still 45 min after peptide injection. Administration of the lower dose of RFRP (1.0 nmol) resulted in a 2.2-fold increase in serum PRL levels that reached statistical significance when compared to hormone levels present in controls at 15 min postpeptide administration. PRL levels present in the 3.0-nmol RFRP treatment group were significantly greater than those in the 1.0-nmol RFRP group 15 and 45 minutes after intracerebroventricular (icv) administration of the peptides ($p < 0.05$). Neither dose of PrRP resulted in significantly altered levels of PRL in serum at any time point when compared with saline-injected controls.

Since RFRP-immunoreactive fibers have been reported to terminate in the vicinity of dopaminergic cells in hypothalamic periventricular and arcuate nuclei *(10)*, we hypothesized that the action of RFRP to stimulate PRL secretion in vivo was a reflection of the peptide's ability to inhibit dopamine (DA) release in the median eminence and thus lower DA levels in the hypophysial portal vessels. To test this hypothesis, we blocked catecholamine synthesis with

**Table 1**
Failure of RFRP-1 to Elevate Serum PRL Levels in Conscious Male Rats Following Catecholamine Synthesis Blockade[a]

| | Serum PRL level (ng/mL, mean ± SEM) | |
|---|---|---|
| Collection time | Saline vehicle controls ($n = 9$) | 3.0 nmol of RFRP-1 ($n = 8$) |
| 0 time (ip injection of α-MPT)[b] | 6.7 ± 1.1 | 6.6 ± 1.6 |
| +90 min (icv injection of saline vehicle or RFRP-1)[c] | 70.7 ± 14.2 | 56.8 ± 11.2 |
| +105 min | 51.9 ± 8.6 | 61.9 ± 12.3 |
| +120 min | 89.2 ± 15.8 | 58.5 ± 12.2 |
| +135 min | 91.0 ± 19.8 | 78.1 ± 21.7 |
| +150 min | 62.3 ± 13.9 | 41.1 ± 9.8 |

[a]All rats received ip α-MPT following 0 time blood sampling, and then icv RFRP-1 (3.0 nmol) 90 min later, followed by four more blood samplings at 15-min intervals.
[b]Following 0 time blood sampling.
[c]Ninety minutes after α-MPT injection.

the enzyme inhibitor α-methyl-para-tyrosine (α-MPT). This treatment resulted in a significant elevation in serum PRL levels, as previously reported by us *(11)*, owing to the withdrawal of DA's inhibitory tone on pituitary PRL release (Table 1). Subequent icv injection of the high dose of RFRP-1 (3.0 nmol), which in control rats resulted in a significant elevation of serum PRL levels, did not significantly alter the already elevated PRL levels. No differences were observed in serum PRL levels at any time point between RFRP-treated and vehicle-injected control animals, all pretreated with the catecholamine synthesis inhibitor.

We employed a second method to determine whether or not the PRL-elevating effect of icv injection of RFRP was due to withdrawal of dopaminergic inhibition of hormone release from the lactotroph *(11)*, blockade of the dopaminergic $D_2$ receptors with domperidone. Administration of iv domperidone resulted in a significant ($p < 0.001$) elevation of serum PRL levels (Table 2), as expected *(11)*. Subsequent icv administration of saline vehicle alone or vehicle containing 3.0 nmol RFRP (the dose that was effective in untreated rats) did not significantly alter the already elevated hormone levels in these animals.

Administration of saline vehicle into the lateral ventricle of conscious male rats, failed to alter serum corticosterone levels (Fig. 3). A significant, dose-related elevation in serum corticosterone levels was observed in rats similarly injected with 1.0 and 3.0 nmol PrRP-31. Corticosterone levels were significantly ($p < 0.01$) elevated, compared to vehicle-injected controls, already at 15 min and remained signifi-

**Table 2**
Failure of RFRP-1 to Elevate Serum PRL Levels in Conscious Male Rats Following Blockade of $D_2$ Dopaminergic Receptors With Domperidone[a]

| | Serum PRL level (ng/mL, mean ± SEM) | |
|---|---|---|
| Collection time | Saline vehicle controls ($n$ = 17) | 3.0 nmol of RFRP-1 ($n$ = 16) |
| 0 time (iv injection of domperidone)[b] | 9.0 ± 1.8 | 8.8 ± 1.5 |
| +45 min (icv injection of saline vehicle or RFRP-1)[c] | 52.4 ± 7.7 | 47.0 ± 8.3 |
| +60 min | 45.5 ± 6.3 | 41.1 ± 5.1 |
| +75 min | 39.3 ± 5.7 | 42.6 ± 10.0 |
| +90 min | 35.5 ± 6.7 | 33.6 ± 7.3 |
| +105 min | 25.3 ± 4.4 | 29.9 ± 5.1 |

[a]All rats received iv domperidone injections following 0 time blood sampling, and then icv saline vehicle or vehicle containing RFRP-1 45 min later. Four additional blood samples were collected at subsequent, 15-min intervals.

[b]Following 0 time blood sampling.

[c]Forty-five minutes after domperidone injection.

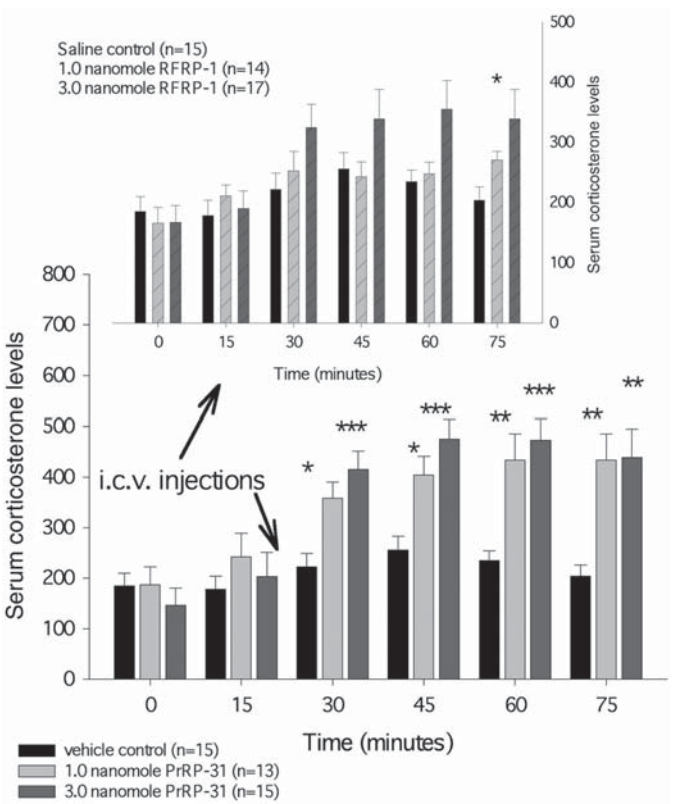


**Fig. 3.** Dose-related effects of central administration of PrRP-31 on serum corticosterone levels in conscious, unrestrained male rats. RFRP-1 administration also resulted in elevated serum corticosterone levels (top), with significant difference present only at the final sampling interval. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs values in saline-injected controls at that time point.

cantly ($p < 0.01$) elevated for the remaining 45 min (60 min after icv administration, compared to saline vehicle–injected controls). A less abrupt rise in serum corticosterone levels was observed in rats administered the lower dose of PrRP-31 (1.0 nmol) with significance first attained 15 min postinjection intracerebroventricularly ($p < 0.05$). Hormone levels remained significantly elevated ($p < 0.05$) compared to controls at 60 min postinjection. When compared to corticosterone levels present in vehicle-injected controls, no significant effects of 1.0 nmol of RFRP-1 were observed at any time point. Corticosterone levels did rise in the high-dose (3.0 nmol of RFRP) group, attaining significance ($p < 0.05$) only at 60 min following peptide administration.

We have previously demonstrated the ability of icv orexin administration *(12)*, in similar doses to those employed here, to effect a significant elevation in serum corticosterone levels in conscious male rats. We were able to block that effect of orexin by pretreatment of the animals with the CRH antagonist *(12)*. We attempted the same blockade strategy with PrRP-31. Pretreatment of conscious male rats with 0.2 mg of CRH antagonist (intravenously) did not alter basal corticosterone levels. Similarly, although this dose of antagonist was sufficient to block the effect of intracerebroventricularly administered orexin *(12)*, it did not significantly alter the corticosterone response to centrally administered PrRP-31 (Table 3). Another strategy we have utilized in the past to block the action of CRH to stimulate ACTH and therefore corticosterone secretion in vivo is passive immunoneutralization *(13)*. Using the same antibodies as those described by us previously, we attempted to block the corticosterone rise to central administration of 3.0 nmol of PrRP-31 (Table 4). Pretreatment with the CRH antiserum did not significantly alter basal corticosterone levels when compared to pretreatment (antiserum given intravenously 90 min earlier) or to levels of hormone present in normal rabbit serum (NRS)–treated controls. However, the serum corticosterone response to icv administration of PrRP-31 (3.0 nmol) was significantly attenuated ($p < 0.01$) in CRH antiserum–treated animals compared with NRS-injected controls receiving the same dose of PrRP-31 (Table 4).

## Discussion

Two members of the RF-amide family of peptides were initially reported to exert significant effects on PRL secretion either in vitro *(1)* or in vivo *(10)*. Cells that produce these peptides innervate hypothalamic sites known to be important in the control of the neuroendocrine response to stress *(3,4,7,8,14,15)*. We hypothesized that these peptides may act in hypothalamus to control the release of PRL and adrenocorticotropin. Furthermore, we hypothesized that these actions were mediated via established neuroendocrine centers within the brain. Initially, we attempted to determine whether these peptides might act directly at the level of the anterior pituitary gland to modify the release of PRL

**Table 3**
Failure of CRH Antagonist to Block Action of icv PrRP-31 to Elevate Serum Corticosterone Levels in Conscious Male Rats[a]

| Collection time | Serum corticosterone level (ng/mL) | |
|---|---|---|
| | Saline vehicle ($n$ = 13) | CRH antagonist ($n$ = 9) |
| 0 time (iv injection of saline vehicle or CRH antagonist)[b] | 257 ± 35 | 187 ± 22 |
| +15 min (icv injection of 3.0 nmol of PrRP-1)[c] | 244 ± 42 | 315 ± 46 |
| +30 min | 352 ± 44 | 412 ± 28 |
| +45 min | 430 ± 52 | 558 ± 63 |
| +60 min | 455 ± 51 | 520 ± 56 |
| +75 min | 466 ± 43 | 521 ± 73 |

[a]CRH antagonist or saline vehicle was injected intravenously following removal of the 0 time blood sample. Fifteen minutes later, following collection of a second blood sample, saline vehicle alone or containing 3.0 nmol of PrRP-31 was injected intracerebroventricularly, followed at 15-min intervals by four more blood collections. Note that although significant elevations in serum corticosterone levels were observed in both pretreatment groups (within-group analysis of variance [ANOVA]), no significant differences were observed between groups at any time point (student's $t$-test).

[b]Following removal of 0 time blood samples.

[c]Fifteen minutes after CRH antagonist or saline vehicle injections.

**Table 4**
Pretreatment with an Anti-CRH Antiserum Significantly Attenuates Corticosterone Response to Central Administration of PrRP-31[a]

| Collection time | Serum corticostero e level (ng/mL) | |
|---|---|---|
| | NRS ($n$ = 21) | anti-CRH antiserum ($n$ = 14) |
| 0 time (iv injection of NRS or anti-CRH antiserum)[b] | 228 ± 39 | 312 ± 54 |
| +90 min (icv injection of 3.0 nmol of PrRP-31)[c] | 254 ± 40 | 275 ± 54 |
| +105 min | 453 ± 23 | 370 ± 33 |
| +120 min | 421 ± 33 | 359 ± 43 |
| +135 min | 316 ± 30 | 303 ± 42 |
| +150 min | 300 ± 42 | 287 ± 43 |

[a]NRS or anti-CRH antiserum was injected intravenously following collection of the 0 time blood sample. Ninety minutes later, following collection of a second blood sample, 3.0 nmol of PrRP-31 was injected intracerebroventricularly in all rats, followed by four additional blood collections at 15-min intervals. Compared to values present just prior to icv PrRP-31 injection, serum corticosterone levels were significantly elevated in the NRS-treated rats 15 ($p < 0.01$) and 30 ($p < 0.05$) min following peptide injection (within-group ANOVA). Similar elevations were not observed in the anti-CRH antiserum–treated rats following icv administration of PrRP-31. When values at each time point were compared between NRS and anti-CRH pretreatment groups, a significant difference in serum corticosterone levels was present 15 min after icv administration of PrRP-31 ($p < 0.05$), reflecting the failure of the hormone to be significantly elevated in the antiserum pretreated rats.

[b]Following 0 time blood sample.

[c]Ninety minutes after NRS or anti-CRH antiserum injection.

and ACTH. PrRP did not significantly alter basal PRL release from cells harvested from male rats, as we have previously reported *(2)*. Similarly, RFRP-1 did not significantly alter PRL release in vitro, a finding that agrees with the results reported in the initial description of this peptide *(10)*. While the discoverers of PrRP maintained that the peptide exerted significant PRL-releasing activity in cells in culture, we have not been able to observe similar effects except when very high concentrations of the peptide (micromolar) are tested and then in cells harvested from female rats *(2)*. Our findings, taken together with the relative absence of PrRP-immunoreactive fibers in the external layer of the median eminence *(3,4,7,8,15)*, led us to conclude that PrRP is not a physiologically relevant neuroendocrine factor controlling PRL release at the pituitary level. It appears that the same can be said of RFRP-1. Finally, neither PrRP-31 nor RFRP-1 significantly altered basal or CRH-stimulated ACTH release from cultures of dispersed anterior pituitary cells. We previously have employed this in vitro paradigm to un-cover the direct pituitary actions of the orexins *(16)* and adrenomedullin *(17)*. Thus, we do not advocate a direct pituitary action of either PrRP or RFRP to control the release of ACTH as well.

We do, however, hypothesize a physiologically relevant action of both peptides to interact with hypothalamic centers important for the control of PRL and ACTH release in vivo. PrRP-31 when injected into the ventricular cavity of conscious male rats resulted in a dose-related, significant elevation in serum corticosterone levels (Fig. 3). This effect was not observed when similar doses of RFRP-1 were administered. These findings suggest an action of PrRP in brain to stimulate the release of CRH into the area of the fenestrated capillary endothelium of the hypophysial portal vessels in the median eminence. If that is the case, then pretreatment of the animals with a CRH antagonist or a neutraliz-

ing anti-CRH antibody might abrogate the central effects of the peptide.

We previously have employed the CRH antagonist in a similar paradigm to demonstrate that centrally administered orexin elevates circulating ACTH and therefore corticosterone levels, by increasing the release of CRH *(12)*. In fact, while these studies were in progress, Matsumoto et al. *(18)* reported that a 10-fold higher dose of the CRH antagonist than the dose employed by us did block the elevation in circulating ACTH levels caused by central administration of PrRP-31. In our hands, unlike our experience in the orexin studies *(12)*, the 0.2 mg/rat dose of antagonist failed to significantly attenuate the corticosterone rise observed following icv administration of PrRP-31 (Table 3). We did not attempt a higher dose of the antagonist because we simply could not afford the expense of the commercially available antagonist, which at current market pricing would have cost $2000 per animal per treatment. Instead, we turned to a second method for the blockade of peptide action that we had previously employed with success *(13)*—passive immunoneutralization. Using the same antiserum previously employed by us to block the in vivo ACTH response to stress *(13)*, we pretreated the rats 90 min prior to central administration of PrRP-31. A significant attenuation of the corticosterone response was observed when compared to levels attained following icv administration of PrRP-31 in rats pretreated with NRS. Thus, we would agree with Matsumoto et al. *(18)* that the ability of PrRP-31 to elevate ACTH secretion is at least in part mediated by an action of the peptide centrally, most likely in the paraventricular hypothalamic area where receptors for the peptide have been localized, to stimulate CRH release. Indeed, Seal et al. *(19)* reported at the time of this article's preparation that PrRP does release CRH from hypothalamic fragments in vitro. It is possible that the peptide also stimulates the release into the portal circulation of additional ACTH-releasing factors, such as oxytocin *(20)*, since others have reported the ability of central PrRP administration to increase circulating levels of that peptide as well *(21)*. We currently are examining that possibility in animals pretreated with either the oxytocin antagonist or our oxytocin antiserum *(22,23)*.

In their initial description of RFRP, Hinuma et al. *(10)* reported that icv administration of a very high dose of RFRP-1 (10 nmol) resulted in a significant elevation in plasma PRL levels in male rats, although it was not stated whether or not the rats were anesthetized at the time of experimentation. A lower dose, 1.0 nmol, did not significantly alter plasma PRL levels. We examined the possibility that PrRP-31 or RFRP-1 might act centrally to elevate circulating PRL levels in conscious, unrestrained male rats. Neither saline vehicle nor PrRP-31 (1.0 or 3.0 nmol) significantly altered serum PRL levels in these animals when injected into the lateral cerebroventricle (Fig. 3). A slight, twofold elevation in serum PRL levels was observed following icv administration of 1.0 nmol of RFRP-1. However, a 5.5-fold increase in serum PRL levels was observed 15 min after icv administration of 3.0 nmol of RFRP-1. This increase attained significance when compared to levels present in vehicle-injected controls at 15 ($p < 0.01$) and 45 ($p < 0.05$) min postinjection.

Hinuma et al. *(10)* had hypothesized that the PRL-releasing action of RFRP was exerted by an action on dopaminergic neurons in the periventricular nucleus where receptors for the peptide had been localized. However, the major dopamineric innervation of the external layer of the median eminence (the tuberoinfundibular DA neurons) originates in the arcuate nucleus, where RFRP-immunopositive nerve fibers also have been identified *(10)*. We hypothesized that by acting on DA neurons, in either location, RFRP would elevate circulating PRL levels by inhibiting the release of DA into the portal vessels. Therefore, we employed an experimental paradigm in which catecholamine synthesis was inhibited, removing the tonic inhibition of the lactotroph in vivo *(11)*, and examined whether RFRP-1 still exerted its PRL-releasing activity. As we have previously described *(11)*, treatment of rats with the catecholamine synthesis inhibitor, α-MPT, resulted in a significant elevation in basal PRL levels in serum. Subsequent, central administration of 3.0 nmol of RFRP-1, under these conditions, failed to stimulate additional PRL release compared to saline vehicle–injected controls (Table 1). This would argue in favor of the Hinuma et al. *(10)* and our hypotheses that the ability of RFRP to elevate PRL levels in vivo is owing to a hypothalamic action to inhibit tuberoinfundibular DA neuron activity and therefore reduce the tonic inhibition of pituitary PRL release. However, it is clear that inhibition of catecholamine synthesis may alter the release of other hypothalamic factors that have been reported to stimulate PRL release. Therefore, we employed a second approach, used by us previously *(11)*, to examine our original hypothesis. Animals were pretreated with an iv dose of domperidone that blocks the $D_2$ dopamine receptor in the pituitary gland. Serum PRL levels were elevated by the receptor blocker and subsequent icv administration of RFRP in a dose that significantly elevated hormone levels in untreated rats failed to exert significant effects on hormone release (Table 2). These data, when taken together with the results of the catecholamine synthesis blockade study, strongly suggest that RFRP acts in hypothalamus to inhibit dopaminergic neuronal activity.

We conclude that endogenous PrRP-31 may act in brain to modulate the release of CRH into the portal vessels and thus play an important role in the hypothalamic control of ACTH and corticosterone secretion in response to stress. It will be important to now compromise PrRP-31 function or production and test the ACTH/corticosterone response to stress in rats. This might establish a physiologically relevant role for the peptide in neuroendocrine function. Similarly, since RFRP acts centrally to stimulate the release of the other major stress hormone, PRL, it is our immediate goal to compromise RFRP function or production in our animals and to examine the physiologic regulation of PRL

secretion. The results presented here are solely pharmacologic in nature. The more difficult task of establishing physiologic relevance remains to be attempted in future studies.

## Materials and Methods

### *Animals*

Intact male Sprague-Dawley (Harlan, Indianapolis, IN) rats (200–250 g) were used in all experiments. All protocols were approved by the institutional animal care and use committee.

### *In Vitro Studies*

Rats were sacrificed by rapid decapitation and anterior pituitary glands were dissected into dispersal medium containing trypsin as previously described *(2)*. Once a single-cell suspension had been obtained, cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) (Biowhittaker, Walkersville, MD) containing 1% penicillin/streptomycin and 10% horse serum (Invitrogen, Carlsbad, CA), aliquoted into 12 × 75 mm polystyrene tubes (about 150,000 per tube), and incubated for 72 h in 5% $CO_2$ at 37ºC. On the day of the experiment, cells were pelleted by centrifugation (10 min, 600*g*, 27ºC), and the medium was removed and replaced by test medium (DMEM, 1% penicillin/streptomycin) containing bovine serum albumin and $2 \times 10^{-5}$ *M* bacitracin (both from Sigma, St. Louis, MO) with either PrRP-31 or RFRP-1 (Phoenix Pharmaceuticals, Belmont, CA). Incubations were conducted for 1 h in 5% $CO_2$ at 37ºC, after which time cells were pelleted as before and medium was stored for assay of PRL and ACTH content by radioimmunoassay (RIA).

### *In Vivo Studies*

All rats were housed individually. An indwelling stainless steel cannula (23 gage, 17 mm) was implanted into the right lateral cerebroventricle under ip tribromoethanol (2.5% in saline, 1.0 mL/100 g of body wt; Sigma) anesthesia. Animals were allowed to return to presurgery body weight (about 5 d) and then tested for cannula patency and placement by icv injection of a dipsogenic dose of angiotensin II *(12)*. Responders were then anesthetized as before and an indwelling jugular cannula was implanted *(24)*. On the following day, rats were moved to a quiet room, and a 12-in. extension tubing was attached to the jugular cannula at its exit on the dorsum of the neck (2 to 3 h after lights on). The cannula was flushed with heparinized saline (250 U/mL of 0.9% NaCl) and animals were left undisturbed for 1 h. In all of the following protocols, blood sampling at each designated time point consisted of the removal of 0.3 mL of whole blood, followed by replacement of the withdrawn volume by an equal volume of isotonic saline (37ºC). Serum was collected by centrifugation and stored frozen until hormone assays were conducted.

#### *Protocol 1*

A second blood sample was collected 15 min after the 0 time sampling. Immediately thereafter icv injections were made via the implanted guide cannula into the lateral cerebroventricle in unrestrained rats. Rats received one of the following injectates: saline vehicle (2 μL), or vehicle containing 1.0 or 3.0 nmol of either PrRP-31 or RFRP-1 (Phoenix Pharmaceuticals). Subsequent blood sampling occurred 15, 30, 45, and 60 min following the icv injections.

#### *Protocol 2*

Following collection of the 0 time blood sample, a dose of the catecholamine synthesis inhibitor α-MPT (200 mg/kg, intraperitoneally; Sigma) was injected to block catecholamine synthesis as previously described *(11)*. Ninety minutes later a second blood sample was collected, after which rats received icv injection of either saline vehicle (2 μL) or vehicle containing 3.0 nmol of RFRP-1. Blood samples were then collected 15, 30, 45, and 60 min after the icv injections were completed.

#### *Protocol 3*

Following collection of the 0 time blood sample, all rats received an iv injection of the $D_2$ dopamine receptor blocker, domperidone (3 mg/kg in 0.2 mL of 0.1 *M* tartaric acid; Janssen) as previously described *(11)*. Forty-five min later a blood sample was collected, followed by icv injection of saline vehicle (2 μL) alone or containing 3.0 nmol of RFRP-1. Blood samples were then collected 15, 30, 45, and 60 minutes after the icv injections were completed.

#### *Protocol 4*

Following collection of the 0 time blood sample, rats received an iv injection of saline vehicle alone (0.2 mL) or the CRH antagonist (α-helical CRH 9-41, 0.2 mg in 0.2 mL of saline; Phoenix Pharmaceuticals) as previously described *(12)*. Fifteen minutes later a second blood sample was collected and then all rats received an icv injection of 3.0 nmol of PrRP-31 (in 2 μL of saline). Blood samples were then collected 15, 30, 45, and 60 min after the icv injections were completed.

#### *Protocol 5*

Following collection of the 0 time blood sample, rats received an iv injection of 0.5 mL of NRS (Sigma) or an equal volume of a polyclonal anti-CRH antiserum previously described by us to significantly attenuate stress-induced ACTH secretion in conscious rats *(13)*. Ninety minutes after serum administration a second blood sample was collected and all rats received an icv injection of 3.0 nmol of PrRP-31 (in 2 μL of saline). Blood samples were then collected 15, 30, 45, and 60 min after the icv injections were completed.

### *Radioimmunoassays*

PRL content of incubation medium and serum was determined by RIA using the kit provided by the National Hor-

mone and Pituitary Program (NHPP, National Institute of Diabetes and Digestive and Kidney Diseases) with the rPRL-RP-3 standard *(25)*. Adrenocorticotropin content of incubation medium was determined using the RIA kit for rat ACTH purchased from Phoenix Pharmaceuticals. Serum corticosterone levels were also determined by RIA (ICN, Costa Mesa, CA).

### *Data Analyses*

Data were analyzed using the SPSS system software. Within- and between-group ANOVA was followed by Scheffe multiple comparison testing. In the data collected from in vivo protocols 2–5, between-group comparisons were conducted using the independent student's *t*-test. Significance was assigned to comparisons that resulted in a probability of occur-rence of $p < 0.05$. All data are presented as group means ± SEM.

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