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Prolactin and 16K Prolactin Stimulate Release of Vasopressin by a Direct Effect on Hypothalamo-Neurohypophyseal System

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Activity of the magnocellular neurons that synthesize vasopressin and oxytocin in the paraventricular and supraoptic nuclei of the hypothalamus can be modulated by local release of neuromediators within the nuclei. Among the bioactive peptides that may play autocrine or paracrine roles in this system is prolactin (PRL). Paraventricular and supraoptic neurons express PRL mRNA and contain and secrete PRL-like proteins of 23 and 14 kDa. We investigated the localization of PRL receptors in vasopressinergic and oxytocinergic magnocellular neurons using dual-label immunofluorescence. The results demonstrate that both vasopressin- and oxytocin-immunoreactive cells of the paraventricular and supraoptic nuclei contain the PRL receptor. In addition, we investigated the possible regulation of vasopressin secretion by PRL using hypothalamo-neurohypophyseal explants in culture. The results show that PRL and a 16 kDa *N*-terminal fragment of the hormone that is analogous to the neurohypophyseal 14-kDa PRL fragment stimulate the release of vasopressin. Together, these findings support the hypothesis that vasopressinergic and oxytocinergic neurons of the magnocellular secretory system are regulated directly by various isoforms of PRL via autocrine/paracrine mechanisms.

Key Words: Prolactin; prolactin receptor; paraventricular magnocellular neurons; supraoptic magnocellular neurons; vasopressin; oxytocin.

Introduction

The hypothalamo-neurohypophyseal system (HNS) consists of magnocellular neurons of the hypothalamic paraventricular and supraoptic nuclei whose axons end in the neurohypophysis. The main neurohormones secreted by these neurons, vasopressin and oxytocin, are transported to the neurohypophyseal nerve terminals, where they are released into the circulatory system *(1)*. Vasopressin plays an important role in cardiovascular regulation and in fluid homeostasis *(2)*, while oxytocin is primarily involved in the regulation of reproductive functions, including parturition, sexual and maternal behavior, and the ejection of milk *(3,4)*.

In addition to vasopressin and oxytocin, other chemical messengers are present in both the soma and the projections of magnocellular vasopressinergic and oxytocinergic neurons (for a review *see* refs. *5* and *6*). Although the physiologic relevance of these molecules remains unclear, a common role suggested is that they regulate the secretion of vasopressin and oxytocin *(6)*. For example, angiotensin II (AII) *(6)* and neuropeptide-Y *(7)* colocalize with vasopressin in magnocellular neurosecretory cells, and both stimulate the release of vasopressin by HNS explants in culture *(8,9)*. Furthermore, the two neurohormones oxytocin and vasopressin can act in an autocrine/paracrine manner to modulate the release of oxytocin *(4,10)* or vasopressin *(11)*, respectively.

Prolactin (PRL)-containing neurons and fibers have been reported in the central nervous system *(12,13)*, including the magnocellular neurosecretory system *(14)*. PRL mRNA is expressed in the isolated paraventricular nucleus (PVN) and supraoptic nucleus (SON) *(14,15)*, and PRL-like immunoreactive and bioactive proteins of 23 and 14 kDa are present in both nuclei and in their axonal projections into the neurohypophysis *(14–17)*. Moreover, immunoreactive PRL is localized within secretory granules in vasopressin-containing magnocellular neurons *(17)* and coreleased with vasopressin in response to high potassium in HNS cultures *(18)*.

However, the functional significance of PRL colocalized with other neurohypophyseal hormones remains unknown. The possibility that PRL isoforms can exert local autocrine/paracrine effects to modulate vasopressin and oxytocin secretion is not unlikely. PRL receptors have been reported in the PVN and SON *(19–21)*, and PRL can stimulate oxytocin mRNA expression *(22)* and oxytocin release *(23,24)* in neurons of these nuclei. However, no effects of PRL on vasopressinergic cells have been reported.

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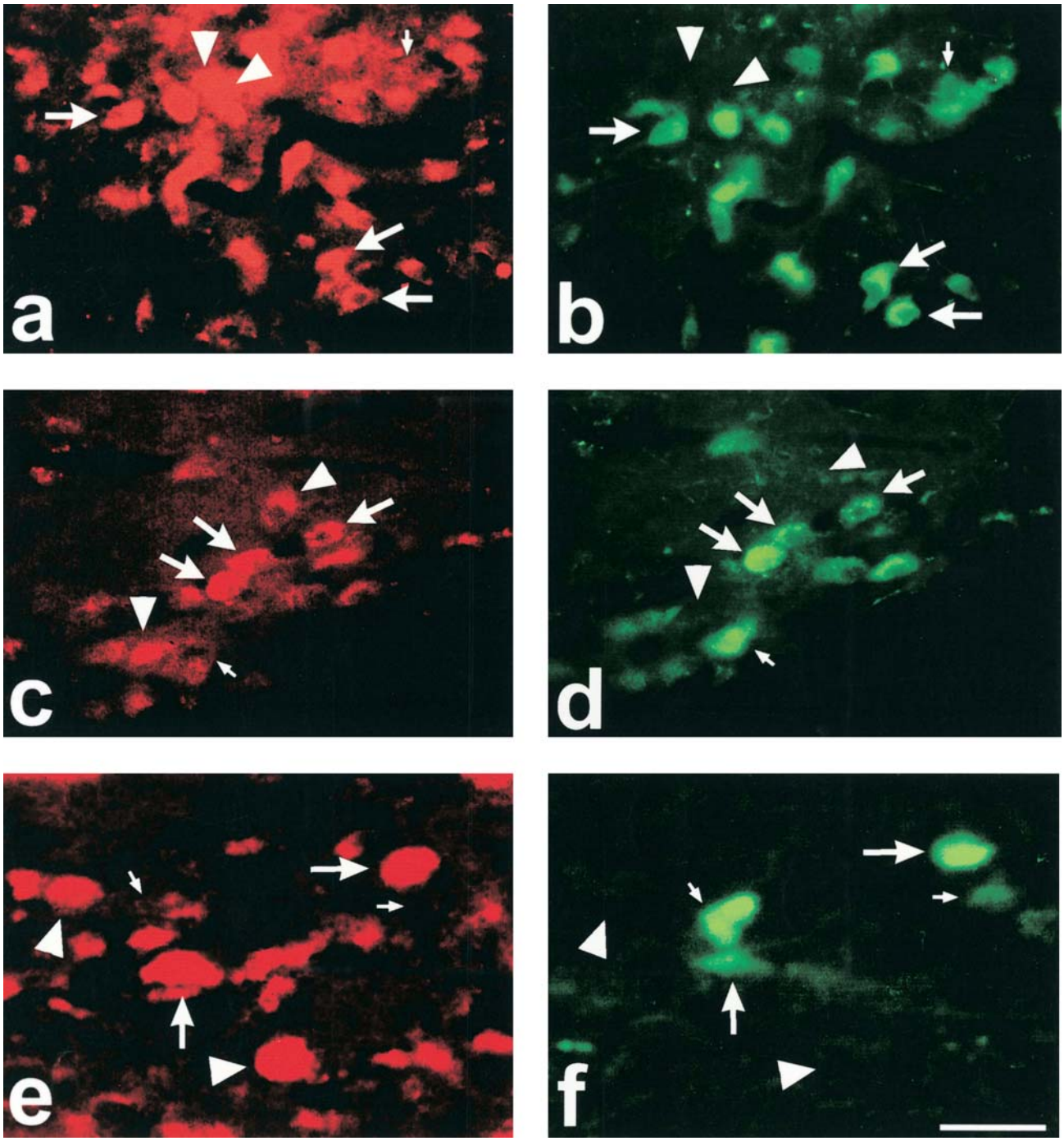

Fig. 1. Double-stained immunofluorescence micrographs of hypothalamus of female rat reacted with anti-PRLR (**a,c,e**) and anti-vasopressin (**b,d**) or antioxytocin (**f**) antibodies. PRLR immunoreactivity was detected in various cell bodies in either the paraventricular (**a,e**) or suproaoptic (**c**) nuclei. Most vasopressin- and oxytocin-immunoreactive cells were positive for the PRLR (large arrows), while few were not (small arrows). There were also PRLR–positive neurons not labeled for vasopressin or oxytocin (arrowheads). Calibration: 50 μm.

In the present study, we examined the cellular colocalization of the PRL receptor (PRLR) with vasopressin and oxytocin in magnocellular neurons of the PVN and SON. We also investigated the effect of PRL and of a 16-kDa fragment of PRL (16K PRL) on the release of vasopressin by HNS explants in culture.

Results

Colocalization of PRLR in Vasopressin and Oxytocin Neurons of PVN and SON

The anti-PRLR antibody (U-5) distinctively labeled cells within both the PVN and the SON (Fig. 1). The lack of sig-

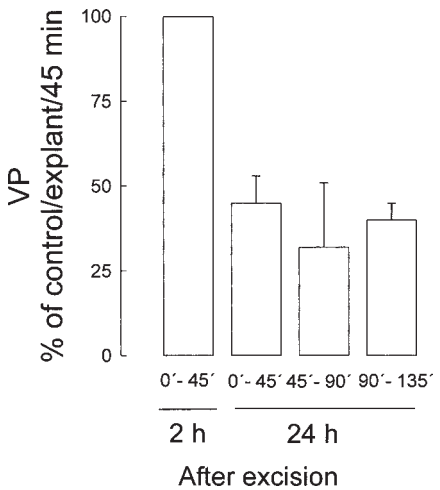

Fig. 2. Spontaneous vasopressin release by cultured hypothalamo-neurohypophyseal explants. HNS explants were cultured individually, and the medium was collected for a 45-min period 2 h after excision and onset of culture, and for three consecutive 45-min periods 24 h after excision. Values are calculated as the percentage of the value (668 ± 45 pg/mL) measured during the initial 45-min period 2 h after explant excision and are expressed as means ± SEM; $n = 6$ for each group.

nificant immunostaining when the antibody was omitted but also when the antibody was substituted by the same concentration of normal mouse IgG established specificity of the antibody reaction. In addition, PRLR-like immunoreactivity was found localized in cells immunostained for vasopressin or oxytocin. The specificity of dual-label immunofluorescence was confirmed by the lack of visible spillover between the two emissions used in the absence of both or either one of the primary antibodies. In the PVN and SON, the population of PRLR-positive cells (Fig. 1a,c,e) outnumbered that of vasopressin- (Fig. 1b,d) or oxytocin- (Fig. 1f) containing neurons. The majority of vasopressin-stained cells were also positive for the PRLR (Fig. 1a–d, large arrows). Likewise, most of the oxytocin-positive cells in both the PVN (Fig. 1e,f, large arrows) and the SON (not shown) contained PRLR-like immunoreactivity. Additionally, there were few vasopressin (Fig. 1 a–d, small arrows) and oxytocin (Fig. 1e,f, small arrows) cells that were not stained for the PRLR, and PRLR-positive cells that did not contain vasopressin or oxytocin (Fig. 1, arrowheads). There was no significant immunostaining for PRLR in the neurohypophysis (not shown).

Vasopressin Release by HNS Explants in Culture

The release of vasopressin from cultured HNS explants decreased over time, reaching stable levels 24 h after excision (Fig. 2). At this time, overall basal vasopressin release from HNS explants (312 ± 51 pg/[explant·45 min]; $n = 44$) was highly variable from explant to explant, but more stable when comparing the secretion from a given explant over three consecutive sampling periods (Fig. 2). Thus, in the following experiments performed to determine the effects of nonspecific (depolarization with high potassium) and specific (AII, 23K PRL, and 16K PRL) stimuli on vasopressin release, the level of vasopressin measured during a 45-min test period 24 h after excision of the HNS is presented as the percentage of the preceding 45-min basal period for the same explant. The validity of this method for the study of the regulation of vasopressin release was assessed by showing that challenge with high potassium (56 m*M*) for 45 min increased the concentration of vasopressin in the medium by 10-fold (Fig. 3A). Returning the concentration of potassium to 5 m*M* during the subsequent 45-min period reduced vasopressin release to levels that were not different from those observed in the initial period. Likewise, AII, a well-known stimulator of vasopressin secretion in vitro *(8)*, significantly increased vasopressin release during the test period (Fig. 3B). This response appears to be dose related, although this relationship is not statistically significant. The statistically significant effects ranged from 50 to 100% stimulation over basal levels, in response to 100 n*M* to 10 μ*M* AII.

Effect of PRL Isoforms on Vasopressin Release by HNS Explants in Culture

Using the explant system, we tested the effects of two isoforms of PRL—the full-length 23-kDa molecule and the *N*-terminal 16-kDa fragment of PRL—on vasopressin release. No effect followed treatment with 0.5 n*M* of either PRL (not shown); however, at higher concentrations both PRL isoforms significantly stimulated the release of vasopressin from HNS explants (Fig. 4). The effects elicited by 5, 50, and 500 n*M* 23K PRL were similar, reaching approx 150% of the basal secretion (Fig. 4). On the other hand, 16K PRL elicited slightly larger stimulations of vasopressin release, ranging from 200 to 300% of the basal values (Fig. 3B).

Discussion

The present study supports the role of PRL molecules in the regulation of the magnocellular neurosecretory system. The results show that PRLRs are colocalized with vasopressin and oxytocin and that PRL isoforms stimulate the release of vasopressin via a direct action on the HNS. This study is the first evidence that PRL has a direct effect on vasopressin neurons.

Numerous putative functions have been attributed to PRL in the brain, including modulation of cerebrospinal fluid composition; mitogenic effects on astrocytes; suppression of fertility; modulation of the stress response; regulation of sleep/wake cycle; and modification of reproductive, parenting, and feeding behavior *(25,26)*. Some of these effects involve regulation of the synthesis and release of various neuropeptides. PRL reduces gonadotropin-releasing hormone gene expression and release *(27)*, inhibits vasointestinal peptide secretion *(24)*, and stimulates the release of hypothalamic corticotropin-releasing hormone *(28)*.

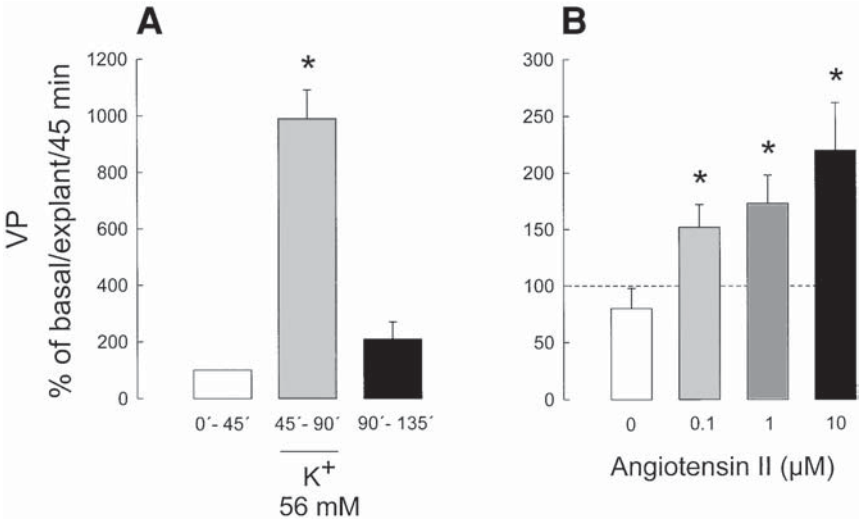

Fig. 3. Vasopressin release by hypothalamo-neurohypophyseal explants in response to K^+-induced depolarization and AII. (**A**) HNS explants were cultured for three consecutive 45-min periods 24 h after excision and onset of culture in Krebs-Ringer buffer (KRB). During the second period, standard KRB was replaced by a KRB containing 56 m*M* K^+, then replaced by standard KRB for the third period. **(B)** Effect of three concentrations of AII. Vasopressin release was determined as shown in (A), then calculated as the percentage of the basal value measured during the initial 45-min period. Values are expressed as means ± SEM; $n = 6$ for each group. *$p < 0.05$ vs basal release rate by analysis of variance (ANOVA).

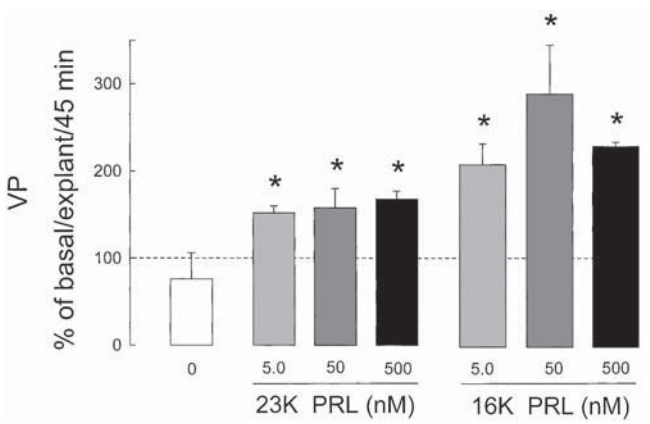

Fig. 4. Vasopressin release by hypothalamo-neurohypophyseal explants in response to increasing doses of 23K or 16K PRL. Values were calculated as the percentage of the basal value measured during the initial 45-min period and are expressed as means ± SEM; $n = 6$ for each group. *$p < 0.05$ vs basal release rate by ANOVA.

In addition, various studies document PRL regulation of the magnocellular neurosecretory system. PRLRs have been demonstrated in the PVN and SON *(19–21)*, and in both nuclei there is an increase in the mRNA *(29)* and in the density *(30)* of PRLRs during lactation. Furthermore, PRL is known to act directly on the HNS to stimulate oxytocin mRNA expression *(22,31)* and oxytocin release *(23,24)*. The physiologic importance of this effect of PRL is suggested by findings showing that suckling-induced release of oxytocin is inhibited when the concomitant release of PRL is prevented by the dopamine agonist bromocriptine or when the action of PRL is antagonized by immunoneutralization *(23)*. This finding implies that stimulation by PRL may be mandatory in order for oxytocin release to occur. However, in contrast to PRL regulation of oxytocin neurons, there is no prior evidence for an action of PRL on vasopressinergic cells.

The present results show that the U-5 anti-PRLR monoclonal antibody (MAb) labels cells within the PVN and SON. This observation confirms the localization of PRLRs in both nuclei previously shown using this and other MAbs *(19,32)* and documented by the autoradiographic localization of ^{125}I-PRL-binding sites *(21)* and the detection of PRLR mRNA by *in situ* hybridization *(21)*. More important, the current study shows, for the first time, that PRLRs colocalize with oxytocin and vasopressin in magnocellular neurons from both the PVN and SON. Although the majority of vasopressin- and oxytocin-positive cells were also stained for the PRLR, there were vasopressin and oxytocin cells that were not positive for PRLRs, as well as PRLR-containing cells that did not stain for vasopressin or oxytocin. These findings support the putative contribution of PRL to central mechanisms operating in the soma of magnocellular neurons to regulate both oxytocin and vasopressin. Besides these mechanisms, there are local controls at the level of the neurohypophysis that amplify or restrain the release of the two neurohormones *(33)*. PRL can also act at the neurohypophyseal level, since it was shown that this hormone stimulates the release of oxytocin by isolated neurohypophyseal lobes *(23)*. However, our study failed to detect PRLR-like immunoreactivity in rat neurohypophyses. This lack of detection is likely owing to a low density of PRLRs rather than

their absence, since the PRLR mRNA has been detected at this site by reverse transcriptase polymerase chain reaction *(34)*.

To investigate a possible effect of PRL that would be consistent with the presence of PRLRs in vasopressin cells, we measured vasopressin release in the presence of PRL by HNS explants in vitro. The explants were found to be suitable because they contained neurons that released vasopressin in response to depolarization with high potassium and to AII, a well-known vasopressin secretagogue *(8,35)*. Two PRL isoforms, the full-length protein (23K PRL) and the *N*-terminal 16-kDa fragment (16K PRL), stimulated the release of vasopressin. Although 16K PRL appeared to evoke a more robust response, the magnitude of the stimulation was not significantly greater than that of PRL. Considering that a lower dose (0.5 n*M*) of PRL and of 16K PRL has no effect, a dose-response stimulation of vasopressin release appears to occur in response to 16K PRL. Nevertheless, as with AII, the high variability in the assay hampers the possible observation of a statistically significant dose-response relationship. The present results are in contrast to previous work performed in lactating rats that failed to show a direct effect of PRL on vasopressin release, both in HNS explants *(22)* and in isolated neural lobes *(23)*. The reasons for these differences are not immediately obvious but may relate to the physiologic state. The high circulating levels of PRL and the increased expression of PRL *(36)* and of PRLRs *(29,30)* in the hypothalamus during lactation could mask the stimulatory action of additional PRL on vasopressin release. Alternatively, methodological differences cannot be disregarded. Our HNS explants contained both the PVN and SON, in contrast to explants in the previous study that contained only the SON *(22)*. We also used a longer stabilization period (18–24 h vs 4 h) than in the earlier experiments, and static incubation in place of perfused incubation *(22)*.

The effect of 16K PRL on vasopressin release may not occur entirely through the activation of PRLRs, because 16K PRL binds only weakly to these receptors *(37)* and has low potency in PRL bioassays *(38)*. In various systems, 16K PRL has unique properties that include inhibition of the formation of new blood vessels *(39)* and proinflammatory actions *(40)*. These functions are mediated via an as-yet unidentified high-affinity site that does not bind 23K PRL *(40,41)*.

The effects of PRL isoforms on the magnocellular secretory system may involve autocrine/paracrine mechanisms. Magnocellular PVN and SON neurons express PRL mRNA *(14,15)*, and PRL-like immunoreactive proteins of 23 and 14 kDa are localized within secretory granules in vasopressin-containing magnocellular neurons *(17)*. Moreover, PRLs are coreleased with vasopressin in response to high-potassium depolarization *(18)*. Presumably, the neurohypophyseal 14-kDa PRL corresponds to the N-terminal part of the PRL molecule, since it is recognized by polyclonal antibodies directed against the N-terminal 16K PRL fragment *(14)* and by MAbs directed against the N-terminal end of PRL *(18)*. This assumption is also consistent with the observation that the neurohypophyseal 14K PRL protein, like 16K PRL, inhibits the proliferation of vascular endothelial cells in culture *(14,16)*.

In conclusion, the present results link PRLs to brain mechanisms regulating vasopressin and oxytocin secretion and action. The functional implications of this connection regarding vasopressin are a matter of speculation. Open possibilities can include the regulation of fluid and electrolyte balance, an important function attributed to both hormones *(2, 42)*. However, high PRL levels associated with PRL-secreting tumors are not known to correlate with inappropriate vasopressin secretion and concomitant water retention and hypoosmolality. Because 16K PRL appears to affect vasopressin with a higher potency than PRL, information about its putative generation in association with prolactinomas and hyperprolactinemia might help resolve this issue.

Materials and Methods

Hormones and Antibodies

Albert F. Parlow of the National Hormone and Pituitary Program (Torrance, CA) donated rat 23-kDa PRL (23 K PRL, biologic grade). 16K PRL was generated after the enzymatic proteolysis of rat 23K PRL with a particulate fraction from rat mammary gland extracts, gel filtration, and carbamidomethylation as reported previously *(43)*. AII was obtained from Calbiochem (La Jolla, CA). Vasopressin was purchased from Sigma (St. Louis, MO) and ^{125}I-labeled vasopressin from Dupont (Boston, MA). MAb directed against the extracellular region of the PRLR (U5) was a kind gift from P. A. Kelly from INSERM U-344 (Paris, France). W. K. Samson kindly provided rabbit polyclonal antisera directed against vasopressin and oxytocin from Saint Louis University (St. Louis, MO).

Animals

Female rats of the Wistar strain (200–250 g) were housed at a constant temperature with a 12:12 h light/dark cycle. They were given free access to water and standard rodent chow. All procedures were reviewed and approved by the institutional Animal Investigation Committee and were in compliance with the National Institutes of Health guidelines.

Simultaneous Double Immunofluorescence

On the day of estrus, determined by daily examination of vaginal smears for two to three consecutive cycles, the animals were anesthetized with sodium pentobarbital (130 mg/kg intraperitoneally) and perfused with 150 mL of phosphate-buffered saline (PBS) pH 7.4, followed by 250 mL of fixative (2% paraformaldehyde and 1.8 g/L of picric acid) prepared in the same buffer. The brain and hypophyseal glands were removed. The hypophyseal neurointermediate lobe was carefully separated from the anterior lobe under a dissecting microscope. Tissues were postfixed for 2 h and then transferred to 20% (w/v) sucrose in PBS until saturation. Both tissues were cut in a cryostat at –20ºC (14-

and 10-µm thickness for brain and neurohypophysis, respectively). Sections corresponding to the anterior hypothalamus (between 900 and 1200 µm caudal to the anterior commissure) were dissected out of other brain areas. These sections and those of the neurohypophyseal gland were mounted on Vectabond-coated slides (Vector, Burlingame, CA) and incubated for 1 h in PBS containing 1% bovine serum albumin (BSA), 10% normal calf serum, and 0.3% Triton X-100. Subsequently, sections were incubated for 72 h in a humid atmosphere at 4ºC with a 1:1000 dilution of the antivasopressin or antioxytocin antisera in combination with 50 mg/mL of the anti-PRLR MAb (U-5). The concentration of U-5 was within the range (1–50 µg/mL) reported for studies in the brain *(32)* and used (20–100 µg/mL) for the localization of PRLRs in other organs *(44)*. All antibodies were diluted in PBS containing 0.3% Triton X-100. The sections were rinsed and incubated for 2 h with goat anti–rabbit IgG antiserum coupled to fluorescein isothiocyanate (FITC) and goat anti–mouse IgG coupled to tetramethyl-Rhodamine (RITC), both from Jackson (Westgrove, PA). Sections were cover-slipped with Aquamont (Vector) and examined by epifluorescence in a Nikon Optiphot epifluorescence microscope equipped with filters for RITC and FITC fluorescence.

Dissection and Culture Explants

Explants of the HNS were dissected from female rats as previously described *(45)* except that the block of tissue was cut at a higher depth to include both the intact PVN and SON together with their axonal projections to the neurohypophysis. Each explant was positioned ventral side down in a plastic well containing 1 mL of F-12K medium supplemented with 20% fetal calf serum, 1 mg/mL of glucose, 200 U/mL of penicillin, and 200 µg/mL of streptomycin (all from Gibco-BRL, Grand Island, NY). For potassium-induced depolarization experiments, HNS explants were cultured in 1 mL of complete KRB (120 m*M* NaCl, 5 m*M* KCl, 2.6 m*M* $CaCl_2$, 1.2 m*M* KH_2PO_4, 0.7 m*M* $MgSO_4$, 22.5 m*M* $NaHCO_3$, 1.8 g/L of glucose, 1.0 g/L of BSA, 0.1 g/L of ascorbic acid, pH 7.4) with or without high potassium (56 m*M*). Medium with high potassium was prepared by decreasing the concentration of sodium by an equimolar quantity. Culture wells were placed in a humidified incubator at 37ºC under 95% air and 5% CO_2. After 18–24 h of stabilization, the incubation medium of HNS explants was changed. Subsequently, the explants were incubated in 700 µL of medium for various (three to eight) consecutive 45-min periods in the presence (test) or absence (basal) of AII, PRL, or 16K PRL at various concentrations. The media were collected and stored at –70ºC until determination of vasopressin by radioimmunoassay (RIA).

Vasopressin RIA

RIA using a method reported earlier *(46)* measured vasopressin content in the medium. All standards and samples were assayed in duplicate. Assay buffer contained 0.01 *M* PBS supplemented with 0.01% BSA and 1:1000 normal rabbit serum, at pH 7.6. Antivasopressin antiserum was used at a final dilution of 1:2000. The assay had a sensitivity of 2 pg/tube.

Statistical Procedures

Because basal vasopressin values varied among the group of explants, the release of vasopressin during the test period was expressed as the percentage of vasopressin released during the preceding basal period for the same explant. Each explant acted as its own control. Differences between basal and test period release of vasopressin were compared by a one-way ANOVA followed by Duncan test for multiple comparisons when appropriate. The limit of significance was $p < 0.05$.

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