Hypocretin/orexin suppresses corticotroph responsiveness in vitro

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Received 21 March 2001; accepted in final form 5 June 2001

Samson, Willis K., and Meghan M. Taylor. Hypocretin/orexin suppresses corticotroph responsiveness in vitro. Am J Physiol Regulatory Integrative Comp Physiol 281: R1140–R1145, 2001.—The hypocretin/orexins (Hcrt/ORXs) are peptides produced in neurons in the lateral hypothalamic area that project to neuroendocrine centers in the hypothalamus. Hcrt/ORX receptors are present in the hypothalamus and anterior pituitary gland. We examined the possibility that the Hcrt/ORXs, which we have demonstrated previously to act in the brain to stimulate sympathetic function, could alter stress hormone secretion by a direct pituitary action. In vitro studies revealed a dose-related inhibitory effect of the Hcrt/ORXs on corticotropin-releasing hormone-stimulated ACTH secretion that appeared to be mediated via the orexin-1 receptor and to be expressed at doses (threshold dose 1 nM orexin A) similar to the affinity constant for the receptor. The effect was not due to abrogation of the cAMP response of the corticotropin to corticotropin-releasing hormone and was not pertussis toxin sensitive, suggesting a non-Gi-mediated mechanism. Instead, a Gq-mediated signaling mechanism was indicated by the protein kinase C blockade with calphostin C to reverse the inhibitory action of orexin A. Orexin A and orexin B did not significantly alter basal ACTH secretion in vitro and did not alter basal or releasing factor-stimulated secretion of luteinizing hormone, prolactin, thyroid-stimulating hormone or growth hormone from cells harvested from male or random-cycle female donors. Our data suggest a direct, pituitary action of the Hcrt/ORXs to modulate the endocrine response to stress and identify the potential cellular mechanism of a unique biological action of the peptides in the anterior pituitary gland. adrenocorticotropin; pituitary; hypothalamus; hypocretin; orexin;

POSTTRANSLATIONAL PROCESSING of the orexin gene product results in the formation of two biologically active peptides (26): the 33-amino acid ORX-A and the 28-amino acid homolog ORX-B. ORX-B was first identified as hypocretin-2 (Hcrt2), and ORX-A is the actual structure of the peptide predicted to be hypocretin-1 (Hcrt1) by De Lecea and colleagues (8). The name hypocretin (Hcrt) was chosen because of the peptides’ hypothalamic localization and their structural similarity to secretin. Sakurai et al. (26) chose the name orexin because of the ability of the peptides to stimulate feeding. Recent reviews of the literature (12, 30) on the Hcrt/ORXs have summarized the findings of numerous groups revealing multiple actions of the peptides, some not directly related to food intake. For example, Hcrt/ORX stimulates arousal (9), activates autonomic outflow from the brain (5, 27, 32), and acts in the brain to inhibit prolactin (PRL) (9, 25) and growth hormone (GH) secretion (9) while stimulating ACTH/corticosterone release (9, 11, 14). Central actions to alter the neuroendocrine regulation of luteinizing hormone (LH) also have been reported (23, 35).

Although production of the peptides is restricted to a discrete population of neurons in the lateral hypothalamus, the projection fields of these neurons have been identified in numerous brain sites, including the nucleus tractus solitarius, locus ceruleus, ventrolateral medulla, raphe, thalamus, septum, and paraventricular and arcuate nuclei of the hypothalamus (7, 20, 22). The existence of Hcrt/ORX-immunopositive nerve terminals in neuroendocrine centers in the hypothalamus (6–8, 10, 20, 22, 26, 37) and the presence there of mRNA encoding both subtypes of the Hcrt/ORX receptors (17, 35) suggested a hypothalamic site of action of the peptides to alter the release of anterior pituitary hormones. In particular, the identification of Hcrt/ORX receptors in the medial parvothalamic portion of the paraventricular nucleus suggested an action of the peptides on corticotropin-releasing hormone (CRH) neurons that project to the median eminence. This would explain the ability of intracerebroventricular injection of a single dose of ORX-A to result in elevated levels of ACTH in plasma (14) and multiple doses of orexin to elevate plasma corticosterone concentrations in two additional studies (9, 11).

Hcrt/ORX-immunopositive fiber staining also has been reported in the median eminence, in particular in the external layer adjacent to the fenestrated capillary endothelium of the hypothalamohypophysial portal vessels (6). This observation, together with the presence of both orexin receptor subtypes in the anterior lobe of the pituitary gland, suggested direct pituitary actions of the peptides. Therefore, we examined the possibility that the Hcrt/ORXs acted as releasing or
inhibiting factors in primary anterior pituitary cell cultures. In particular, we hypothesized that the actions of the Hcrt/ORXs in the brain to stimulate CRH release (11) and activate autonomic function (27, 32) were complemented by direct pituitary actions on the release of the stress hormones PRL and ACTH.

MATERIALS AND METHODS

Intact male and random-cycle female rats (200–250 g; Harlan Sprague Dawley, Indianapolis, IN) were killed by decapitation as approved by the university animal care committee. Anterior pituitary glands were collected and mechanically dispersed in the presence of trypsin, as previously described (31). Cells were then aliquoted into 12 × 75 mm polystyrene tubes (~200,000 cells/tube for ACTH protocols) and incubated for 24 h or into 24-well plates (~300,000 cells/well) and incubated for 72 h in medium 199 (pH 7.3) containing 20 mM HEPES, 10% horse serum, and 1% antibiotic-antimycotic (all from GIBCO-BRL, Grand Island, NY) in room air at 37°C. All protocols were conducted in minimal volumes separate harvest populations of cells. On the day of experimentation, cells were washed with fresh medium after removal of culture medium from plated cells or after centrifugation (660 g, room temperature, 10 min) of cell suspensions and exposed for 1 h to peptides diluted in test medium (0.8 ml of medium 199 containing 20 mM HEPES and 1% penicillin-streptomycin (all from GIBCO-BRL) and 0.1% BSA and 0.02 nM bacitracin (both from Sigma Chemical, St. Louis, MO)).

Because we did not know the actual amount of orexin peptide present in the normal rat anterior pituitary gland or the levels present in hypophysial portal plasma, we initially employed a very wide log molar dose range of peptides in our examination of the potential effects on basal and releasing factor-stimulated hormone secretion. In one protocol, cells were pretreated with pertussis toxin (PTX, 100 ng/ml) for 18 h before exposure to test medium containing peptides. PTX catalyzes ADP-ribosylation of guanine nucleotide-binding regulatory proteins, and, specifically, if PTX blocked the inhibitory effect of orexin on CRH-stimulated ACTH secretion, a mechanism of action of orexin via interaction with a Gαi protein would be indicated (see DISCUSSION). In another protocol, the protein kinase C (PKC) inhibitor calphostin C or PTX blocked the effect of PTX on basal ACTH release (11) and activate autonomic function (27, 32) and 11.2 ± 1.4 pg ACTH with 0.1 nM ORX-A and in control, respectively) was dose related and significant at 1.0 nM (Fig. 2). In the same cell harvests, the effect of ORX-B was much less impressive, not reaching significance until doses as high as 100 nM (Fig. 2).

Accumulation of cAMP in response to CRH, in the absence or presence of IBMX (1 mM added 30 min before the peptides), was not significantly altered by coincubation with 100 nM ORX-A (Table 3). Similarly, in additional cell harvests, ORX-A by itself did not alter basal cAMP accumulation: 2.1 ± 0.1 (control),

Table 1. Failure of ORX-A to alter basal or releasing factor-stimulated GH, PRL, or LH secretion from cultured anterior pituitary cells

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>ORX-A</th>
<th>RF</th>
<th>ORX-A + RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>6.4 ± 0.5</td>
<td>7.4 ± 0.5</td>
<td>55.4 ± 7.0</td>
<td>49.3 ± 2.7</td>
</tr>
<tr>
<td>(TRH)</td>
<td>(TRH)</td>
<td>(TRH)</td>
<td>(TRH)</td>
<td>(TRH)</td>
</tr>
<tr>
<td>PRL</td>
<td>5.5 ± 0.4</td>
<td>5.5 ± 1.0</td>
<td>12.0 ± 1.4</td>
<td>13.0 ± 0.9</td>
</tr>
<tr>
<td>(TSH)</td>
<td>(TSH)</td>
<td>(TSH)</td>
<td>(TSH)</td>
<td>(TSH)</td>
</tr>
<tr>
<td>TSH</td>
<td>48 ± 3</td>
<td>40 ± 6</td>
<td>94 ± 19</td>
<td>96 ± 14</td>
</tr>
<tr>
<td>(GnRH)</td>
<td>(GnRH)</td>
<td>(GnRH)</td>
<td>(GnRH)</td>
<td>(GnRH)</td>
</tr>
<tr>
<td>LH</td>
<td>0.19 ± 0.06</td>
<td>0.20 ± 0.02</td>
<td>4.9 ± 1.0</td>
<td>4.6 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as nanograms of hormone released from male donor rats; n = 6. ORX-A, orexin A (100 nM); RF, releasing factor (10 nM); GH, growth hormone; PRL, prolactin; TSH, thyroid-stimulating hormone; LH, luteinizing hormone; GnRH, GH-releasing hormone; TRH, thyrotropin-releasing hormone; GnRH, gonadotropin-releasing hormone.

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1.9 ± 0.1 (1.0 nM ORX-A), 2.0 ± 0.1 (10 nM ORX-A), and 2.0 ± 0.2 (100 nM ORX-A) pmol cAMP accumulated (n = 5). The inhibitory effect of ORX-A on CRH-induced ACTH secretion was not prevented (Fig. 3) by PTX treatment (18 h, 100 ng/ml). In contrast, the PKC inhibitor calphostin C (100 nM) significantly reversed the inhibitory effect of ORX-A (Fig. 4).

DISCUSSION

One of the classic neuroendocrine markers of stress is ACTH secretion, and this phenotypic response can be regulated at several anatomic sites, including the hypothalamus (i.e., control of CRH release into the median eminence) and the anterior pituitary gland (i.e., corticotroph response to CRH). In addition to a possible hypothalamic site of action of Hcrt/ORX, the presence of receptors for the peptides in the anterior lobe suggests biological actions in the pituitary gland itself.

Our initial hypothesis was that Hcrt/ORX exerted effects in the anterior pituitary gland that reflected general arousal and perhaps even stress. This hypothesis was based on the reported presence of the orexin-1 receptor (OX1R) and, to a lesser degree, the orexin-2 receptor (OX2R) in the anterior lobe of the pituitary gland and the abundance of peptide in nerve terminals in the median eminence (6). Because orexin receptors had not been localized to any one particular cell type in the gland, we examined the potential for an effect on the secretion of several hormones in vitro. We expected that the peptides might stimulate ACTH and PRL secretion, both hormones being released during stress. No significant effects of ORX-A on basal or TRH-stimulated PRL release were observed. This finding was corroborated during the conduct of our experiments by preliminary findings from another group (25).

Neither basal nor releasing factor-stimulated LH, TSH, or GH secretion from cells in culture was ob-

Table 2. Failure of ORX-A to significantly alter basal ACTH release from cultured pituitary cells harvested from intact male or random-cycle female rats

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>ACTH Released, pg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male donors</td>
</tr>
<tr>
<td>Control</td>
<td>19.5 ± 1.0</td>
</tr>
<tr>
<td>ORX-A</td>
<td></td>
</tr>
<tr>
<td>0.1 pM</td>
<td>15.9 ± 1.4</td>
</tr>
<tr>
<td>1.0 pM</td>
<td>18.0 ± 1.2</td>
</tr>
<tr>
<td>10 pM</td>
<td>20.7 ± 2.0</td>
</tr>
<tr>
<td>100 pM</td>
<td>16.1 ± 1.5</td>
</tr>
<tr>
<td>1.0 nM</td>
<td>15.9 ± 0.9</td>
</tr>
<tr>
<td>10 nM</td>
<td>19.8 ± 1.7</td>
</tr>
<tr>
<td>100 nM</td>
<td>18.7 ± 1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6, except male controls, n = 12.

Table 3. Failure of ORX-A to alter basal or CRH-stimulated cAMP accumulation in IBMX-treated, cultured anterior pituitary cells harvested from adult male rats

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>CRHstimulated cAMP Accumulated, pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without ORX-A</td>
</tr>
<tr>
<td>Control</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>CRH</td>
<td></td>
</tr>
<tr>
<td>0.01 nM</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>0.1 nM</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>1.0 nM</td>
<td>2.9 ± 0.1†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6. CRH, corticotropin-releasing hormone; IBMX, 3-isobutyl-1-methylxanthine. *P < 0.05; †P < 0.01 vs. control and/or without ORX-A.
served to be significantly affected by ORX-A. Thus the reported effects of centrally administered orexin on LH (23, 35) secretion may not be due to diffusion of the centrally administered peptide into the hypophysial portal circulation but, instead, as hypothesized by others, may be due to neuromodulatory actions in the diencephalon. However, our in vitro studies were conducted with cells harvested from male and random-cycle female rat donors. It is still possible that significant effects on LH or PRL secretion might be detected in cells harvested from “staged” animals in distinct phases of the estrous cycle, but certainly in cells harvested from male rats no significant effects were observed. A hypothalamic action of Hcrt/ORX to alter TRH release can be hypothesized, since in our hands ORX-A did not alter basal or TRH-stimulated TSH release in vitro. Although levels of orexin in peripheral plasma are very low (2), no report has been published on levels of the peptide in hypophysial portal plasma. Alternatively, because nerve fibers staining positive for orexin are present in the neurohypophysis (6), the peptide may be carried in the short portal vessels that connect the posterior pituitary with the anterior lobe. ORX-B content of the pituitary gland and median eminence actually exceeds that of ORX-A (6). However, peptide content as determined by RIA may be misleading if the two peptides have different half-lives or are “cleared” by different mechanisms in vivo. The metabolic fate of the two peptides, once released in these tissues, has not been reported.

A consistent finding was the ability of Hcrt/ORX to significantly decrease the magnitude of CRH-induced ACTH section in vitro. The effect of ORX-A occurred in our cell cultures at doses that are similar to the calculated affinity constant (EC\textsubscript{50} = 30 nM) for ORX-A binding to OX\textsubscript{1R} expressed in Chinese hamster ovary cells (26). In our studies, ORX-A was more potent than ORX-B, suggesting an action via the OX\textsubscript{1R} receptor (26) in nontransfected normal cells. Indeed, OX\textsubscript{1R} is more abundant than OX\textsubscript{2R} in the rat anterior pituitary according to one report (6). After the original version of this manuscript was submitted for publication, it was reported (3) that the ORX receptor in the human adenohypophysis is localized to the somatotrophs and corticotrophs. We would speculate then that the effect of orexin to inhibit CRH-induced ACTH release that we observed in our rat pituitary cell cultures might also be expressed in the human pituitary. We cannot speculate on the significance of the orexin receptor on human somatotrophs, since in our rat cell cultures, orexin did not alter basal or GHRH-stimulated GH secretion.

If these in vitro data have physiological significance, then the source of endogenous Hcrt/ORX would, in all likelihood, be the Hcrt/ORX-producing neurons in the lateral hypothalamus that project to the median eminence (6). One group failed to detect by RIA immunoreactive orexin in extracts of rat pituitary gland (34); however, Date and colleagues (6) detected immunoreactivity in the gland, albeit at levels 20-fold lower than those detected in the adjacent median eminence. Thus Hcrt/ORX may access the anterior lobe via the long portal vessels of the hypothaloohypophysial vasculature, acting as a true neuroendocrine hormone. Although levels of orexin in peripheral plasma are very low (2), no report has been published on levels of the peptide in hypophysial portal plasma. Alternatively, because nerve fibers staining positive for orexin are present in the neurohypophysis (6), the peptide may be carried in the short portal vessels that connect the posterior pituitary with the anterior lobe. ORX-B content of the pituitary gland and median eminence actually exceeds that of ORX-A (6). However, peptide content as determined by RIA may be misleading if the two peptides have different half-lives or are “cleared” by different mechanisms in vivo. The metabolic fate of the two peptides, once released in these tissues, has not been reported.
We endeavored to determine the mechanism by which Hcrt/ORX exerts its inhibitory effect on the corticotroph, reasoning that ORX-A might interrupt the cAMP-mediated signaling cascade that translates the effect of CRH. However, doses of ORX-A that significantly inhibited CRH-induced ACTH secretion did not, by themselves, alter cAMP accumulation and did not significantly interrupt the accumulation of cAMP in response to CRH stimulation. These observations were made in the absence and presence of the phosphodiesterase inhibitor IBMX, and thus it is unlikely that orexin alters adenyl cyclase activity (i.e., formation of cAMP) or termination of the cAMP signal (i.e., inactivation by phosphodiesterase). Furthermore, PTX pretreatment did not abrogate the effect of ORX-A. Our results do not support a Gs-mediated action of ORX-A to inhibit CRH-induced ACTH secretion. Similarly, in cultured hypothalamic neurons, cells that respond to hypocretin with an increase in cytosolic calcium levels (37), cAMP levels did not rise in the presence of effective doses of the peptide. Van den Pol et al. (37) also ruled out a role for Gs in the mechanisms of action of hypocretin in their system. They did suggest, however, a role for a Gq protein in the action of hypocretin in hypothalamic neurons, since a PKC inhibitor was able to block the rise in cytosolic calcium in response to hypocretin (37). It appears from our data that a Gq-mediated mechanism of action of ORX-A exists in the anterior pituitary gland as well. We were able to reverse the inhibitory effect of ORX-A on CRH-induced ACTH secretion with calphostin C, a PKC inhibitor that acts by blocking the enzyme’s binding site for diacylglycerol (13). Exactly where in the orexin receptor-phospholipase C-PKC signaling cascade ORX-A acts is a current focus of this laboratory. Clearly, it does not act via PKC-mediated inhibition of cAMP phosphodiesterase activity (1). There was a trend toward increased ACTH release in response to CRH in the presence of calphostin C; however, at this dose of blocker, significance was not obtained. Because others have reported that activation of PKC augments ACTH secretion in response to arginine vasopressin (1, 16), the orexins must be affecting some signaling pathway downstream from PKC other than the inhibition of phosphodiesterase activity. Also, orexin may not be activating the specific PKCs that are linked to phosphodiesterase activity (18).

In the brain, orexin acts to apparently stimulate CRH release (11) and to activate the autonomic nervous system (27, 32), both actions related to the physiological response to stress. In the pituitary gland, an opposite effect on ACTH secretion is exerted, and the physiological relevance of these observations awaits further study. It will be important to learn the significance of the OX1R as in the pituitary gland, and although a naturally occurring OX2R gene deletion has been reported (15), a unique OX1R gene deletion or compromise has not been reported. Similarly, specific high-affinity antagonists for OX1R and OX2R have not been reported, although recently a moderate-affinity antagonist has been reported (24, 33). That genetic model (OX1R knockout) and those pharmacological tools (antagonists) would greatly facilitate future studies.

The gene deletion for the orexin peptides has been reported (4); however, it is not known whether those animals experience altered responses to stress or if they demonstrate compromised/exaggerated adrenal function that may be related to a loss of the potential inhibitory effects of orexin in the pituitary gland. In addition, because loss of hypocretin neurons has been hypothesized to be a basis for the human condition of narcolepsy (21), it would be of interest to determine in narcoleptic patients whether their hypothalamo-pituitary-adrenal axis remains competent during the onset and progression of the disease. It is not clear, however, that the level of disruption of production of hypocretin in the brains of narcoleptic individuals would affect a potential action of the peptide in the pituitary gland, since it is not known whether pituitary exposure to hypocretin, from hypothalamic or intrapituitary sources, is compromised or whether sensitivity to the peptide (if indeed the same actions can be demonstrated in humans as in our rat cell cultures) is altered in this condition.

**Perspectives**

We do not suggest that the inhibitory effect of orexin that we describe here is a major determinant of ACTH secretory dynamics. Indeed, there are several other endogenous neuropeptides that also “modulate” the action of CRH (28, 29). One, adrenomedullin, inhibits basal as well as CRH-stimulated ACTH release (29); another, proadrenomedullin NH2-terminal 20 peptide, inhibits CRH-stimulated, but not basal, corticotropin secretion (28). Non-cAMP-dependent mechanisms of action have been hypothesized for the effects of adrenomedullin and proadrenomedullin NH2-terminal 20 peptide on the corticotroph, similar to the conclusion we have now made for Hcrt/ORX. However, the potential pituitary actions of the Hcrt/ORXs should be recognized as possible side effects if or when Hcrt/ORX analogs are tested for clinical efficacy, and the potential for the Hcrt/ORXs to play a physiologically significant role in the regulation of corticotroph function deserves further study.

The gift of RIA reagents by the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases) and A. F. Parlow is gratefully acknowledged.

These studies were supported by the Max Baer Heart Fund from the Fraternal Order of Eagles and by funds provided by St. Louis University.

**REFERENCES**


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Lu XY, Bagnol D, Burke S, Akil H, and Watson SJ. Differential distribution of 
OX1 and 


