The effect of D-aspartate on luteinizing hormone-releasing hormone, α-melanocyte-stimulating hormone, GABA and dopamine release

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Since D-aspartate stimulates prolactin and LH release, our objective was to determine whether D-aspartate modifies the release of hypothalamic and posterior pituitary factors involved in the control of their secretion and whether its effects on these tissues are exerted through NMDA receptors and mediated by nitric oxide. In the hypothalamus, D-aspartate stimulated luteinizing hormone-releasing hormone (LHRH), α-melanocyte-stimulating hormone (α-MSH) and GABA release and inhibited dopamine release through interaction with NMDA receptors. It increased nitric oxide synthase (NOS) activity, and its effects on LHRH and hypothalamic GABA release were blunted when NOS was inhibited. In the posterior pituitary gland, D-aspartate inhibited GABA release but had no effect on dopamine or α-MSH release. We report that D-aspartate differentially affects the release of hypothalamic and posterior pituitary factors involved in the regulation of pituitary hormone secretion. *NeuroReport* 13:1–4 © 2002 Lippincott Williams & Wilkins.

Key words: D-Aspartate; Hypothalamus; Neuropeptides; Neurotransmitters; Posterior pituitary

INTRODUCTION

D-Aspartate is an endogenous D-amino acid that participates in the regulation of several endocrine gland functions in mammals. In the pituitary gland, it has been localized in lactotropes of the anterior lobe [1], in nerve processes and terminals of the neural lobe and in scattered cells in the intermediate lobe [2]. D-Aspartate is also localized in most magnocellular neurons of the supraoptic and paraventricular nuclei and in the median eminence [2,3]. Its localization in different areas of the hypothalamic–pituitary axis suggested that D-aspartate might have a role in neuroendocrine modulation. In fact, D-aspartate has been shown to increase serum growth hormone (GH), luteinizing hormone (LH) and prolactin levels [4,5]. We have reported that this amino acid has a direct stimulatory effect on prolactin release from anterior pituitary cells [6].

The regulation of prolactin and LH release from the anterior pituitary is complex and involves several inhibitory and stimulatory factors, including luteinizing hormone-releasing hormone (LHRH), α-melanocyte-stimulating hormone (α-MSH), GABA and dopamine [7,8]. Since i.p. administration of D-aspartate increases prolactin and LH release [4,5], the aim of the present study was to investigate the effect of D-aspartate on the release of these factors from the hypothalamus and the posterior pituitary.

D-Aspartate has high affinity for the glutamate binding site of NMDA receptors [9] and may act through their activation [4,10]. The activation of NMDA receptors induces, among other responses, an increase in nitric oxide synthase (NOS) activity in hypothalamic neurons [11]. Hence, another goal of this study was to evaluate whether the effects of D-aspartate in the hypothalamus and the posterior pituitary are exerted through NMDA receptor activation and whether they involve nitric oxide synthesis.

MATERIALS AND METHODS

Male Wistar rats (200–250 g body weight) were housed in controlled temperature and light conditions. Food and water were available *ad lib*. After decapitation, the posterior pituitary gland (intermediate and neural lobes) was removed and a hypothalamic explant including the arcuate and periventricular nuclei, the medial preoptic area and the median eminence was dissected.

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Tissues were incubated in a Dubnoff shaker at 37°C, in 95% O_2/5% CO_2 with 0.5 ml Krebs–Ringer bicarbonate buffer (KRB) pH 7.4, containing 10 mM glucose, 10 mM Hepes, 1 mM ascorbic acid, 0.1 mM bacitracin and 0.1% bovine serum albumin (BSA). Incubations were performed in the presence of magnesium as described by Kubrusly *et al.* [10].

One hypothalamic explant was incubated for the determination of LHRH concentration and one hypothalamic explant or one posterior pituitary gland was incubated for the determination of α-MSH concentration. Tissues were allowed to equilibrate for 15 min and the medium was discarded. The tissues were incubated for 30 min with fresh KRB (basal release). At the end of the incubation period, the medium was aspirated and media and tissues were frozen. The media were heated for 5 min in a 100°C water bath and centrifuged at 10 000 r.p.m. for 10 min. The supernatants were stored at −70°C until determination of LHRH and α-MSH by RIA. LHRH was measured with a specific LHRH antiserum kindly provided by Ayala Barnea (University of Texas Southwestern Medical Center, Dallas, USA) using [^{125}I]LHRH as tracer. The sensitivity of the assay was 0.2 pg/tube, and the curve was linear up to 100 pg LHRH. α-MSH was determined by the method of Eberle [12], who kindly provided α-MSH antibody, using [^{125}I]α-MSH as tracer. The sensitivity of this assay was 4 pg/tube, and the curve was linear up to 1000 pg/tube. The intra- and interassay coefficients of variation for both assays were < 10%.

For GABA determination, the tissues were preincubated for 15 min and then incubated for 30 min; this medium was discarded. Next, they were incubated for 30 min in 40 mM K^+ medium (prepared by substituting NaCl on a molar basis; evoked release). At the end of this incubation period, the media were aspirated and media and tissues were frozen. The media were heated for 5 min in a 100°C water bath and centrifuged at 10 000 r.p.m. for 10 min. GABA concentration was determined by the [^{3}H]muscimol radioreceptor assay described by Bernasconi *et al.* [13] (sensitivity range 12.5–200 pmol/ml).

For the determination of dopamine concentration, two hypothalamic explants or three posterior pituitaries were preincubated in KRB containing 0.01 mM L-tyrosine for 15 min and then for another 30 min (basal release). Hypothalamic explants were further incubated for 30 min in 40 mM K^+ medium (evoked release). The substances to be tested were present during both periods of incubation. After the incubation period, the media were collected in 0.1 M $HClO_4$ with 0.65 mM $Na_2S_2O_5$ and immediately frozen on dry ice. Next, the media were centrifuged at 15 000 r.p.m. for 10 min and the supernatants stored at −70°C until dopamine determination by HPLC with electrochemical detection, as described previously [14]. Briefly, dopamine was extracted from the incubation media with 2 M Tris–HCl, pH 8.7 and acid alumina using 3,4-dihydroxybenzylamine hydrobromide (DHBA) as internal standard. After washing, the pellets were resuspended in 0.4 M acetic acid. The supernatants were filtered and injected into an analytical column (Luna 5 μm, C18, 4.6 × 250 mm, Phenomenex) kept at 37°C. The mobile phase was prepared with 100 mM NaH_2PO_4, 1 mM heptanesulfonic acid, 0.5 mM EDTA and 6% acetonitrile, pH 3.0.

NOS activity was determined by conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline, using a modification of the method described by Bredt *et al.* [15].

All tissues were homogenized in cold distilled water, and protein concentration in the homogenates was determined by the method of Lowry *et al.* using BSA as standard.

[^{125}I]radionucleide, [^{3}H]muscimol and solvable were purchased from New England Nuclear , Boston, MA, USA. Dowex AG 50W-X8 resin was purchased from Bio-Rad Laboratories, Hercules, CA, USA, [^{14}C]L-arginine from Amersham Corp., Buckinghamshire, UK and scintillation cocktail from Wallac Oy, Fin/Fisher Chemicals, UK. D-Aspartic acid (D-aspartate), DL-2-amino-5-phosphonovaleric acid (AP-5), N′-nitro-L-arginine methyl ester (L-NAME) and all other drugs were purchased from Sigma-Aldrich Co., USA.

Data are expressed as means ± s.e.m., and were analyzed by the unpaired Student's *t*-test, one-way ANOVA followed by Dunnett's test for comparisons against the control group or Student–Newman–Keuls multiple comparisons test for multiple comparisons, or two-way ANOVA with interaction terms. Differences with $p < 0.05$ were considered statistically significant. All experiments were performed at least twice. Figures represent results of individual experiments.

RESULTS

D-Aspartate increased LHRH and α-MSH release from hypothalamic explants. These effects were reversed by a competitive NMDA receptor antagonist, AP-5 (Fig. 1). D-Asparate also stimulated K^+-evoked GABA release and inhibited K^+-evoked dopamine release (Table 1), but did not modify basal dopamine release (data not shown). The effects on K^+-evoked GABA and dopamine release were blocked by AP-5 (Table 1). Since our results suggest that D-aspartate affects hypothalamic dopamine release through NMDA receptors, we tested whether NMDA affects dopamine release from hypothalamic explants. In effect, NMDA (0.1–1 mM) decreased K^+-evoked dopamine release (control 1.71 ± 0.09 ng/mg protein; 0.1 mM NMDA 1.25 ± 0.09; 1 mM NMDA 1.14 ± 0.03; $p < 0.01$; $n = 5$) without modifying basal dopamine release (data not shown).

Considering that the effects of D-aspartate on the release of LHRH, α-MSH, GABA and dopamine from hypothalamic explants seem to be exerted through NMDA receptors, and given that NMDA receptor activation stimulates hypothalamic NOS activity [10], we examined the effect of D-aspartate on NOS activity in the hypothalamus. D-Aspartate increased NO production in hypothalamic explants as assessed by conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline (control 20.5 ± 0.6 d.p.m./μg protein; 1 mM D-asp 23.5 ± 1.2; $p < 0.05$; $n = 8$). Supporting the participation of NO in the effect of D-aspartate on LHRH release, L-NAME, a NOS inhibitor, impeded the D-asp-induced increase in LHRH release (Table 2). Similarly, D-aspartate failed to modify K^+-evoked GABA release in the presence of this inhibitor (Table 2).

In the posterior pituitary gland, D-aspartate did not significantly modify α-MSH release (control 1.14 ± 0.25 μg/mg protein; 1 mM D-aspartate 0.73 ± 0.12;

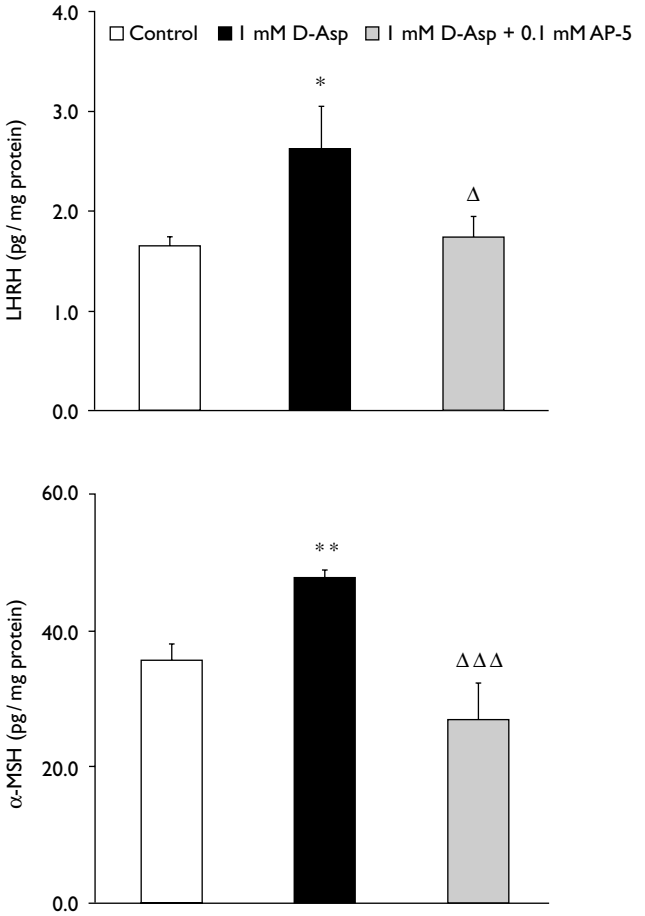

Fig. 1. Effect of D-aspartate on LHRH and α-MSH release from hypothalamic explants. Values represent mean ± s.e.m. ($n = 4$–10 per group). Data were evaluated by one-way ANOVA, followed by Student–Newman–Keuls multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* control; $\Delta p < 0.05$, $\Delta\Delta\Delta p < 0.001$ *vs* 1 mM D-aspartate.

Table 1. Effect of D-aspartate on K^+-evoked GABA and dopamine release from hypothalamic explants.

	GABA (nmol/mg protein)	Dopamine (ng/mg protein)
Control	3.49 ± 0.32 (6)	1.24 ± 0.08 (5)
1 mM D-aspartate	6.63 ± 0.89 (8)*	0.80 ± 0.08 (6)*
1 mM D-aspartate + 0.1 mM AP-5	2.83 ± 0.42 (6)**	1.22 ± 0.10 (6)**

Values represent mean ± s.e.m. (*n* per group). Data were evaluated by one-way ANOVA, followed by Student–Newman–Keuls multiple comparisons test.
*$p < 0.05$ *vs* control;
**$p < 0.01$ vs 1 mM D-aspartate.

$n = 6$–7). It decreased K^+-evoked GABA release from this tissue, an effect not reversed by AP-5 (Fig. 2). Although NMDA decreased basal dopamine release from the posterior pituitary, D-aspartate did not modify it (control 0.50 ± 0.06 ng/mg protein; 0.1 mM NMDA 0.07 ± 0.03; $p < 0.01$; 1 mM D-aspartate 0.53 ± 0.04; $n = 4$–5).

Table 2. Effect of D-aspartate on LHRH and evoked GABA release from hypothalamic explants in the presence of a NOS inhibitor.

	LHRH (pg/mg protein)	GABA (nmol/mg protein)
Control	2.34 ± 0.27 (5)	3.54 ± 0.82 (6)
0.5 mM L-NAME	1.31 ± 0.18 (6)	5.62 ± 1.98 (4)
0.5 mM L-NAME + 1 mM D-aspartate	1.98 ± 0.38 (6)	4.38 ± 0.42 (5)

Values represent mean ± s.e.m. (*n* per group). Data were evaluated by one-way ANOVA.

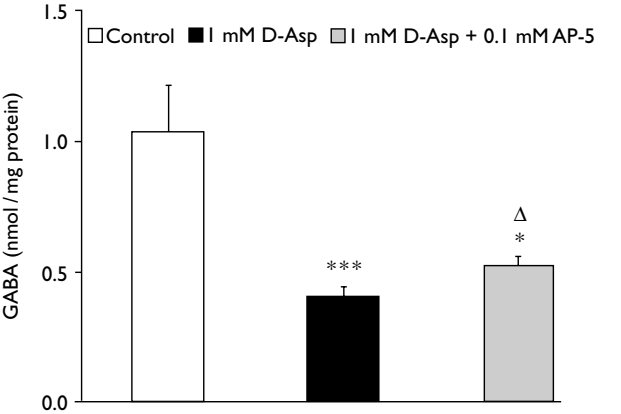

Fig. 2. Effect of D-aspartate on K^+-evoked GABA release from the posterior pituitary gland. Values represent mean ± s.e.m. ($n = 7$–8 per group). Data were evaluated by one-way ANOVA, followed by Student–Newman–Keuls multiple comparisons test. $^{*}p < 0.05$, $^{***}p < 0.001$ *vs* control; $\Delta p < 0.05$ *vs* 0.1 mM D-aspartate.

DISCUSSION

Our findings show that D-aspartate affects the release of some of the neuropeptides and neurotransmitters involved in the regulation of prolactin and LH secretion. D'Aniello *et al.* showed that D-aspartate stimulated LHRH release and suggested that this increase was responsible for the rise in serum LH observed following i.p. administration of this compound [4]. Our data confirm the stimulatory effect of D-aspartate on LHRH release and show that this effect is exerted through binding to NMDA receptors, since it was blocked by AP-5, a competitive NMDA receptor antagonist which does not affect LHRH release *per se* [16].

NOergic neurons of many hypothalamic nuclei express NMDA receptors [17], and several reports indicate that NO mediates the stimulatory effect of NMDA receptor activation on LHRH release [16,18]. Our results show that D-aspartate increases NOS activity in the hypothalamus. Tallying with this fact, D-aspartate did not affect LHRH release when NOS activity was inhibited, suggesting that NO mediates the increase in LHRH release induced by D-aspartate. The increase in NO triggered by D-aspartate

may have a dual action, since NO stimulates LHRH release [18] whereas it increases GABA release, which accounts for the termination of the LHRH pulse [19]. Our data also show that D-aspartate increases hypothalamic α-MSH release through NMDA receptor activation. Wayman *et al.* reported that NMDA stimulates α-MSH release from the hypothalamus by a mechanism involving NO production [20]. Therefore, it is possible to speculate that the effect of D-aspartate on α-MSH release could also be mediated by NO.

NO inhibits hypothalamic dopamine release [21] and induces GABA release [19]. Abreu *et al.* demonstrated that peroxynitrites generated by the reaction between NO and superoxide inhibit the activity of tyrosine hydroxylase, the rate-limiting enzyme in the biosynthesis of catecholamines, in the median eminence [22]. In our study, D-aspartate decreased dopamine release whereas it increased GABA release from the hypothalamus. The rise in NO synthesis induced by D-aspartate may inhibit dopamine release and also stimulate GABA release, which in turn may inhibit tuberoinfundibular dopaminergic activity [23]. The decrease in hypothalamic dopaminergic tone could lead to an increase in prolactin release, reinforcing the direct stimulatory action of D-aspartate on lactotropes [6]. Our results show that the inhibitory effect of D-aspartate on dopamine release is reversed by a NMDA antagonist. Also, NMDA has an inhibitory effect on hypothalamic dopamine release, thus supporting the hypothesis of the participation of NMDA receptors in the effects of D-aspartate on dopamine release from the hypothalamus.

The present study shows that the effects of D-aspartate at the hypothalamic level are exerted through NMDA receptors. However, in the posterior pituitary gland the inhibitory effect of D-aspartate on GABA release was not reversed by a NMDA receptor antagonist, suggesting that it was not exerted through this receptor subtype. On the contrary, we have shown that NMDA receptor activation stimulates GABA release from this tissue [24]. Several studies have indicated that some effects of D-aspartate could be independent of glutamate receptor activation [25,26]. For example, Yuzaki *et al.* found evidence of a putative aspartate receptor in Purkinje cells whose electrophysiological pattern differs from that of NMDA receptors [26]. Therefore, the effects observed on GABA release from the posterior pituitary gland may be exerted mainly via an unknown receptor with affinity for D-aspartate. The decrease in GABA release to the short portal vessels induced by D-aspartate could contribute to the stimulatory effect of D-aspartate on prolactin release [6].

CONCLUSION

D-Aspartate acts differentially at the hypothalamic and posterior pituitary levels. At the hypothalamic level, its actions on the neuropeptide and neurotransmitter release studied are mediated by NMDA receptors and involve NO production, but are not exerted through NMDA receptor activation at the posterior pituitary level. Its effects at these two levels could contribute to an increase in prolactin and LH release from the anterior pituitary.

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