Burmese Russell’s viper venom causes hormone release from rat pituitary cells in vitro

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REVISED MANUSCRIPT RECEIVED 27 February 1989

ABSTRACT

Acute and chronic hypopituitarism is associated with severe envenoming by the Burmese Russell’s viper. We have demonstrated that in vitro, Burmese Russell’s viper venom (0.1–10 μg/ml) causes a dose-dependent release of GH, TSH and ACTH from dispersed rat anterior pituitary cells in culture. At 10 μg/ml, venom causes a significant increase in the release of GH (344%, P<0.001), TSH (168%, P<0.005) and ACTH (>700%, P<0.001). We have also shown that the component (or components) responsible for this stimulatory effect is stable to heat (60°C, 1 h) and mild trypsinization. Repeated addition of venom (1 μg/ml) to pituitary cells in a perifusion column system demonstrated attenuation of GH release. This reduced response was not due to depletion of the GH pool since the pituitary cells were subsequently able to respond to both GH-releasing factor (GRF) stimulation and KCl depolarization. Somatostatin in a dose which abolished GRF-stimulated GH release failed to affect venom-stimulated GH release, implying that venom acts in a cyclic AMP-independent manner. We conclude that Burmese Russell’s viper venom has direct effects on pituitary hormone release in vitro. Whether these effects contribute to its known actions in vivo on the function of the pituitary remains to be established.

Journal of Endocrinology (1989) 122, 489–494

INTRODUCTION

Acute and chronic pituitary failure has been described in patients envenomed by the Burmese Russell’s viper. Clinical features of hypopituitarism, such as lack of response of plasma growth hormone (GH) to insulin-induced hypoglycaemia, were documented in 30% of patients who had apparently recovered from snake bites (Tun-Pe, Warrell, Tin-Nu-Swe et al. 1987). Chronic hypopituitarism has also been described in three patients bitten by Russell’s vipers in India (Eapen, Chandy, Kochuvarky et al. 1976).

In Burma, pituitary haemorrhage was a frequent necropsy finding in patients who died after envenoming (Aung-Khin, 1977). This description of haemorrhagic necrosis of the pituitary has led to the suggestion that the pituitary failure seen in these patients resembles that of post-partum pituitary necrosis (Sheehan’s syndrome). In this syndrome, post-partum haemorrhage, with its attendant shock and frequent coagulation abnormalities, is thought to precipitate ischaemic necrosis (Sheehan, 1940).

Russell’s viper venom contains well-characterized procoagulants, and defibrination is a hallmark of systemic envenoming (Myint-Lwin, Warrell, Phillips et al. 1985). Tun-Pe et al. (1987) have suggested that local vascular occlusion by fibrin could be a major cause of the pituitary lesion, with venom haemorrhagins also contributing by damaging pituitary capillary endothelium.

Animal venoms are known to consist of a heterogeneous mixture of substances including peptides. For example, several neurotoxins have been isolated from bee venom, some of which have been shown to produce membrane depolarization (Cherubini, Ben Ari, Gho et al. 1987) and selective blockage of Ca2+-activated K+ channels (Hugues, Romey, Duval et al. 1982). We have therefore investigated the possibility that Burmese Russell’s viper venom might have direct effects on the release of GH, TSH or ACTH from dispersed pituitary cells. For these in-vitro studies we have used dispersed rat anterior pituitary cells in a perifusion system or as monolayer cultures.

MATERIALS AND METHODS

Preparation of rat anterior pituitary cells

Anterior pituitary cells were obtained from male Wistar rats (180–200 g). Pituitary glands were collected
into phosphate-buffered saline (PBS; 7·4 mmol Na₂HPO₄/l; 0·7 mmol KH₂PO₄/l; 0·15 mmol NaCl/l; pH 7·3) and divided into small (1 mm) pieces. The tissue was incubated at 37°C with 0·125% (w/v) trypsin and 0·02% (w/v) EDTA in PBS. After 15 min the tissue was centrifuged (2000 g, 4 min) and resuspended in fresh trypsin solution. This procedure was repeated three times and followed by two 20-min incubations with 0·15% (w/v) collagenase (Boehringer-Mannheim Ltd, London, U.K.) in PBS. The dispersed cells were washed twice in PBS and resuspended in either Earle's balanced salt solution (EBSS; Gibco, Paisley, Strathclyde, U.K.) for perfusion experiments or in Earle's minimal essential medium containing 10% (v/v) heat-inactivated fetal calf serum (Gibco), 100 U penicillin/ml, 100 mg streptomycin/ml (Glaxo, Greenford, Middx, U.K.) and 2·5 mg fungizone/ml (Gibco), buffered with Hepes, hereafter referred to as culture medium for monolayer culture experiments. Cell viability determined by trypan blue exclusion was > 90%. The average yield per pituitary was 1·3 million cells.

Materials

Burmese Russell’s viper venom (Sigma Chemical Co., Poole, Dorset, U.K.) was initially dissolved in normal saline and further diluted in either EBSS or culture medium. This venom was used in all experiments described. Pure lyophilized venom from a Burmese Russell’s viper was also provided by Dr R. D. G. Theakston, Liverpool School of Tropical Medicine, Liverpool, U.K. Synthetic rat corticotrophin-releasing factor (CRF), human GH-releasing factor (1–44) (GRF(1–44)), thyrotrophin-releasing hormone (TRH) and somatostatin (SRIF; Sigma Chemical Co.) were prepared in lyophilized form (Adams, Bhuttacharji, Halliwell et al. 1984) and dissolved directly into EBSS or culture medium before each experiment.

Cultured pituitary cells

Dispersed pituitary cells suspended in culture medium were divided into multiwells (Gibco), each well containing 1 ml culture medium and 0·3–0·4 million cells. Cultures were initially incubated at 37 °C for 2 days to allow for cell attachment before the experiments. Experiments were carried out at 37 °C with randomization of cultures for each study. At least triplicate cultures were used for the controls and each variable. Cells were washed with fresh culture medium before each experiment. The cultures were incubated for 4 h in 1 ml culture medium (basal period). The cells were then washed and incubated for the same time-period (experimental period) in fresh medium with GRF, CRF, TRH or venom, or without any additive (control experiment). Media were stored at −20 °C before use. Results were expressed as means ± S.E.M. of the percentage of hormone secreted by cells in the experimental period compared with that produced by the same cells in the preceding basal period. The significance of the data was determined by Student’s t-test.

Perifused pituitary cells

A heterogeneous population of dispersed pituitary cells (4–9 million cells per column) was mixed with Bio-Gel P₃ resin beads (Bio-Rad, Watford, Herts, U.K.) and perifused (1 ml/min) with EBSS containing 2·5 mg BSA/ml as described previously (Yeo, Thorner, Jones et al. 1979). In some experiments, two columns were perifused simultaneously. The number of cells added to paired columns varied by less than 5%. After 60 min of perifusion with medium alone, each column was pulsed with test substance or vehicle for 2 min with a period between each successive pulse of at least 20 min. Fractions (2 ml) were collected from each column and stored at −20 °C before hormone analysis by radioimmunoassay as described below.

Radioimmunoassay

Concentrations of rat GH, TSH and ACTH in the media were measured by direct double-antibody techniques with reagents kindly provided by the NIAMMD, Bethesda, MD, U.S.A. Mean inter- and intra-assay coefficients of variation were, respectively 13·2 and 10·0% for GH, 12·0 and 12·0% for TSH and 12·5 and 9·0% for ACTH.

RESULTS

Pituitary cell culture studies

Burmese Russell’s viper venom (0·1–10 μg/ml) caused a dose-dependent increase in GH, thyroid-stimulating hormone (TSH) and adrenocorticotrophin (ACTH) secretion after a 4-h incubation (Fig. 1). Venom, at a concentration of 0·1 μg/ml, had no significant effect on GH secretion. However, higher concentrations of venom (1 and 10 μg/ml) stimulated GH release by 192% (P<0·02) and 344% (P<0·001) respectively, compared with the basal value. Similarly, venom caused a significant increase in ACTH release at concentrations of 1 μg/ml (301%, P<0·02) and 10 μg/ml (>700%). Venom was a less potent stimulator of TSH release, but caused a significant increase of 168% (P<0·005) at the highest concentration used (10 μg/ml). GRF (2 nmol/l) significantly stimulated GH release by 424% (P<0·001); CRF (2 nmol/l)
significantly stimulated ACTH release by 570% \((P<0.001)\), and TRH \((20 \text{ nmol/l})\) significantly stimulated TSH release by 179% \((P<0.002)\) over the same incubation period.

Venom \((10 \mu\text{g/ml})\) subjected to heat treatment or mild trypsinization still retained its ability to stimulate pituitary GH, TSH and ACTH secretion, with no significant loss in activity (Fig. 2).

![Figure 1](image1.png)

**Figure 1.** Effect of increasing concentrations of venom \((0.1-10 \mu\text{g/ml})\) on GH, TSH and ACTH release from cultured rat anterior pituitary cells. Hormone secretion in the 4-h experimental period is expressed as a percentage of that in the previous 4-h basal period. Values are means ± S.E.M. of quadruplicate cultures in a single experiment, and are representative of data from three such experiments. Hormone release stimulated by 2 nmol GH-releasing factor (GRF)/l, 20 nmol thyrotrophin-releasing hormone (TRH)/l and 2 nmol corticotrophin-releasing factor (CRF)/l is also shown. C, control. *\(P<0.02\), **\(P<0.001\) compared with the basal value (Student's \(t\)-test).

![Figure 2](image2.png)

**Figure 2.** Effect of untreated venom (UTV; 10 \(\mu\text{g/ml}\)), heat-treated venom (HTV; 60 °C, 1 h) \((10 \mu\text{g/ml})\) and venom subjected to mild trypsinization (TTV; 12.5 mg trypsin/ml, 37 °C, 1 h) \((10 \mu\text{g/ml})\) on pituitary GH, TSH and ACTH release. Hormone secretion in the 4-h experimental period is expressed as a percentage of that in the previous 4-h basal period. Values are means ± S.E.M. of triplicate cultures in a single experiment and are representative of data from three such experiments. C, control. There were no significant differences between heat- or trypsin-treated venoms and untreated venom (Student's \(t\)-test).

Venom \((10 \mu\text{g/ml})\) had no significant effect on cell viability, compared with medium alone. Cell viability, after a 4-h incubation, was >90% in experiments both with and without venom, as assessed using trypan blue exclusion.
Perifusion studies

The effect of three repeated additions of Burmese Russell's viper venom on GH release from perifused rat pituitary cells is shown in Fig. 3. Basal GH release averaged 100 μg/l. GRF (2 nmol/l) stimulated GH release by 1120%, and the initial addition of venom (1 μg/ml) stimulated GH release by >1600%. However, a sharp reduction in the GH response to the second and third additions of venom was observed. Although the GH response to this venom became attenuated, GRF (2 nmol/l) and KCl (56 nmol/l), added at the end of the experiment, stimulated GH release by 1100% and >1600% respectively.

The effect of SRIF (10 and 100 ng/ml) on venom-stimulated GH release from perifused rat pituitary cells is shown in Fig. 4. Basal GH release without the addition of SRIF averaged 100 μg/l. An initial perifusion with GRF (1 nmol/l) caused a 600% increase in GH release from both columns. The subsequent two additions of GRF (1 nmol/l), which also stimulated GH release by approximately 600%, were completely abolished by SRIF (10 and 100 ng/ml respectively). The first addition of venom (1 μg/ml) caused a 10000% increase in GH release, which was not affected by SRIF (10 ng/ml). Subsequent additions of venom, which exhibited the previously noted reduced stimulatory effect on GH release, were also not affected by SRIF (100 ng/ml). Results were repeated using the pure preparation of Burmese Russell's viper venom and equivalent results obtained.

DISCUSSION

In the present study, cultured rat anterior pituitary cells were highly responsive to increasing concentrations of venom (0.1–10 μg/ml), which produced a rise in GH, ACTH and TSH release. Such a non-specific, stimulatory effect on pituitary hormone release could be due to a cytotoxic action of the venom, causing cell death and massive hormone release. However, this appears unlikely in view of the fact that the cell viability, determined by trypan blue exclusion, after exposure to venom (10 μg/ml) for 4 h was >90%. The ability of both GRF and KCl to stimulate GH release from perifused cells previously exposed to three separate additions of venom, also indicates that the stimulatory action of venom is not due to its cytotoxic effects. Indeed, Chaim-Matyas & Ovadia (1987) have recently shown that Russell's viper venom (6 μg/ml) is not toxic to melanoma cells in vitro. The acute stimulation of all three pituitary
hormones measured, strongly suggests a non-specific effect of the venom on hormone release. However, it is not yet clear whether this effect of the venom is limited to the pituitary or whether it affects other cell types.

Results from our perfusion studies show that the repeated addition of venom caused a reduction in the GH response. The limited capacity of the anterior pituitary to respond to continuous administration of GRF has been documented in vivo (Wehrenberg, Ling, Brazeau et al. 1982) and in vitro (Bilezikjian & Vale, 1984; Ceda & Hoffman, 1985; Bilezikjian, Seifert & Vale, 1986). This 'desensitization' has been thought to be partly due to the depletion of the pool of releasable GH, a marked decrease in pituitary GH content being seen after prolonged exposure to GRF in vitro (Ceda & Hoffman, 1985). However, our results (Fig. 3), also show that the pituitary cells retain their full ability to respond to GRF and KCI, even though the GH response to venom has been attenuated. This would indicate that the reduction in the GH response to venom is not due to GH pool depletion. It has been reported that the decrease in apparent sensitivity to GRF may be due to the down-regulation of GRF binding sites (Bilezikjian et al. 1986) and/or an impairment of the coupling of a component of the adenylate cyclase system (Simard & Labrie, 1986). However, the ability of GRF to stimulate GH release after desensitization with venom suggests that the venom is impairing an alternative mechanism.

Somatostatin, the physiological inhibitor of GH release, abolished GRF-stimulated GH release from perfused pituitary cells, but had no effect on venom-stimulated GH release (Fig. 4). The inhibition of GH release by SRIF has been shown to be, at least in part, due to its inhibitory effect on adenylate cyclase (Bilezikjian & Vale, 1983; Harwood, Grew & Aguileria, 1984). This would also suggest that Russell's viper venom can stimulate GH release via a mechanism which is cyclic AMP-independent.

Our results clearly show that the component (or components) of Russell's viper venom responsible for the stimulation of pituitary GH, TSH and ACTH release is resistant to both heat treatment and mild trypsinization. Russell's viper venom has been shown to contain several isoenzymes of phospholipase A2 (Salach, Turini, Seng et al. 1971) which may influence membrane fluidity and receptor-effector coupling. Phospholipase A2 from snake venoms is remarkably stable to heat treatment (Deems & Dennis, 1981) and trypsinization (De Haas, Slotboom, Bonsen & Von Deenan, 1970). However, whether a phospholipase is responsible for the stimulatory effects of Russell's viper venom on pituitary hormone release is not yet known.

In conclusion, it appears that a heat-stable component (or components) of Burmese Russell's viper venom can stimulate GH, ACTH and TSH release from cultured rat pituitary cells in a dose-dependent manner. Whether this acute stimulation of pituitary hormone release is eventually responsible for the in-vivo findings of acute and chronic hypopituitarism in patients sustaining severe envenomation (Eapen et al. 1976; Tun-Pe et al. 1987), remains to be established.

ACKNOWLEDGEMENTS

We thank the NIAMMD for the supply of reagents for the rat pituitary hormone assays. A pure preparation of Burmese Russell's viper venom was kindly provided by Dr R. D. G. Theakston. We are also greatly indebted to Dr Tha Aung, Institute of Medicine, Rangoon, Burma for obtaining and forwarding an initial supply of venom from which the final analytical methods were evolved.

REFERENCES


