Desensitization of the adrenocorticotrophin responses to arginine vasopressin and corticotrophin-releasing hormone in ovine anterior pituitary cells

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Abstract

Following repeated or prolonged exposure to either corticotrophin-releasing hormone (CRH) or arginine vasopressin (AVP), pituitary adrenocorticotrophin (ACTH) responsiveness is reduced. This study compared the characteristics of desensitization to CRH and AVP in perfused ovine anterior pituitary cells. Desensitization to AVP occurred at relatively low AVP concentrations and was both rapid and readily reversible. Treatment for 25 min with AVP at concentrations greater than 2 nM caused significant reductions in the response to a subsequent 5 min 100 nM AVP pulse ($IC_{50} = 6.54$ nM). Significant desensitization was observed following pretreatment with 5 nM AVP for as briefly as 5 min. Desensitization was greater following a 10 min pretreatment, but longer exposures caused no further increase. Resensitization was complete within 40 min following 15 min treatment with 10 nM AVP. Continuous perfusion with 0.01 nM CRH had no effect on AVP-induced

desensitization. Treatment with 0.1 nM CRH for either 25 or 50 min caused no reduction in the response to a subsequent 5 min stimulation with 10 nM CRH. When the pretreatment concentration was increased to 1 nM significant desensitization was observed, with a greater reduction in response occurring after 50 min treatment. Recovery of responsiveness was progressive following 50 min treatment with 1 nM CRH and was complete after 100 min. Our data show that in the sheep AVP desensitization can occur at concentrations and durations of AVP exposure within the endogenous ranges. This suggests that desensitization may play a key role in regulating ACTH secretion *in vivo*. If, as has been suggested, CRH acts to set corticotroph gain while AVP is the main dynamic regulator, any change in responsiveness to CRH may significantly influence the overall control of ACTH secretion.

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Introduction

Regulation of adrenocorticotrophin (ACTH) secretion during stress is a multi-factorial process, with the hypothalamic neuropeptides corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) being the most physiologically important ACTH secretagogues (Antoni 1993). Both hormones are secreted into the hypophyseal portal circulation in a pulsatile fashion (Engler *et al.* 1989, Antoni 1993) and stimulate ACTH secretion from pituitary corticotrophs via activation of distinct G protein-coupled receptors (De Keyser *et al.* 1994). The relative importance of CRH and AVP in the physiological regulation of ACTH secretion remains a matter of debate. The ability of the two hormones to stimulate ACTH secretion appears to vary depending on the species. In both the rat (Vale *et al.* 1983) and horse (Evans *et al.* 1993) CRH is a much more potent secretagogue than AVP, whereas in the

sheep some groups, but not all, have reported that AVP is the more potent secretagogue (Familiari *et al.* 1989, Evans *et al.* 1993, McFarlane *et al.* 1995). In all cases a marked synergism between CRH and AVP has been observed (Antoni 1986). It has been proposed that this synergism may allow CRH to function as a permissive signal, setting the overall responsiveness of the corticotroph, while AVP is the main dynamic hypothalamic ACTH-releasing factor (Antoni 1993, Evans *et al.* 1996). Studies in the rat have shown an increase in the expression of AVP in parvocellular neurons during stress and good correlation in general between changes in the number of AVP receptors and ACTH responsiveness (Aguilera 1994). This suggests that AVP is an important modulator of ACTH responsiveness during stress.

In vivo studies have shown that the ACTH responses to both AVP and CRH undergo regulatory changes during chronic stress (Aguilera 1994). Additionally, prolonged

infusion of either CRH (Wynn *et al.* 1988) or AVP (Antoni *et al.* 1985, Koch & Lutz-Bucher 1985) results in down-regulation of their respective receptors. *In vitro* studies have shown that prior exposure to either AVP or CRH can result in a reduction in ACTH response, or desensitization, to a subsequent stimulation with the same neuropeptide (Reisine & Hoffman 1983, Holmes *et al.* 1984, Hoffman *et al.* 1985, Evans *et al.* 1988, 1993, 1996, Thomson *et al.* 1990, Castro 1993). The treatments used in these *in vitro* studies to induce desensitization were either of high concentration, long duration or a combination of both and did not closely match the characteristics of endogenous CRH and AVP pulses. The following examples illustrate this. Treatment of rat anterior pituitary segments with 100 nM AVP for 4 h greatly reduced their responsiveness to a subsequent stimulation with either 10 nM or 1 µM AVP (Holmes *et al.* 1984). Similarly a 1 h treatment with 100 nM CRH caused substantial desensitization to a subsequent stimulation with 100 nM CRH (Reisine & Hoffman 1983). In perfused sheep anterior pituitary cells repeated stimulation with 10 min AVP pulses (100 or 2000 nM) every 60 min for 4 h reduced the ACTH responsiveness of the cells, and successive 10 min pulses of CRH (0.2–2000 nM) also caused decreased ACTH release with time (Evans *et al.* 1988).

In contrast to these *in vitro* treatments, endogenous CRH and AVP pulses are brief and of relatively low maximal concentration. *In vivo* studies have provided information on the pituitary portal characteristics of pulses of CRH and AVP under basal and stressed conditions in sheep (Caraty *et al.* 1988, 1990, Engler *et al.* 1989, Battaglia *et al.* 1998). Basal secretion rates were very low for both AVP and CRH (e.g. ranging between 5 and 83 pM for AVP and between 1 and 19 pM for CRH) (Caraty *et al.* 1988). Exposure to a stressor resulted in pulses of secretion of AVP and CRH which usually lasted less than ~30 min. The maximal AVP concentration in portal plasma following exposure to a stressor ranged from ~1 nM for acute haemorrhage (Caraty *et al.* 1988) to ~6 nM for high-dose insulin-induced hypoglycaemia (2 IU/kg) (Caraty *et al.* 1990). For CRH the maximal portal plasma concentrations were between ~0.2 nM for acute haemorrhage (Caraty *et al.* 1988) and ~0.4 nM for insulin-induced hypoglycaemia (2 IU/kg) (Caraty *et al.* 1990) (our conversion of all data). Therefore it is very unlikely that corticotrophs would be exposed to CRH or AVP at either the levels or for the lengths of time that have been used, in general, to induce desensitization *in vitro*. As a result the question remains as to whether desensitization seen under experimental conditions plays a role in the physiological regulation of ACTH secretion, or reflects pharmacological actions of the regulators.

The main aim of the current study was to determine whether desensitization occurs *in vitro* in ovine anterior

pituitary cells in response to CRH and AVP treatments which more closely match the concentrations and durations of CRH and AVP pulses observed in the hypophyseal circulation of the sheep. By investigating desensitization under these more physiologically relevant conditions we were able to make a better assessment of the role of these processes in the regulation of ACTH secretion. Secondly, in view of the hypothesis that CRH acts as a permissive signal setting the overall responsiveness of the corticotroph we assessed the effect of treatment with CRH on the ability of AVP to induce desensitization to a subsequent stimulation. Finally, we wanted to establish the relationship between the degree of desensitization and pulse duration and magnitude, and to determine the time required for resensitization to occur, since these properties may give some clues as to the mechanisms involved in the regulation of the receptor.

Materials and Methods

Cell preparation

Pituitary glands were collected from a local abattoir in accordance with animal ethics laws of New Zealand and the guidelines of the University of Canterbury. Dispersed ovine anterior pituitary cells were prepared as previously described (Evans *et al.* 1985, Le Beau & Mason 1994). Briefly, whole pituitary glands were removed from sheep shortly after slaughter and placed into cold, sterile Hepes buffer (25 mM Hepes, 137 mM NaCl, 5 mM KCl, 10 mM glucose and 0.002% phenol red, pH 7.3) containing antibiotic and antimycotic agents (100 U penicillin/ml, 100 mg streptomycin/ml and 0.25 mg amphotericin B/ml). A suspension of dispersed cells from the combined anterior lobes of seven pituitaries was obtained following a combination of enzymatic (collagenase type II, 480 U/ml Hepes buffer; Sigma Chemical Co., St Louis, MO, USA) and mechanical disruption. The dispersed cells were washed by centrifugation (300 g for 5 min at 4 °C) and resuspension in Hepes buffer, and after the final wash were resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM); Sigma) containing 3.7 mg NaHCO₃/ml, 584 mg L-glutamine/ml, non-essential amino acids, antibiotic/antimycotic agents (as above) and 25 mM Hepes at pH 7.3, and supplemented with 10% new-born calf serum (NCS) (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY, USA). For perfusion experiments 4.5×10^6 cells were plated into non-adherent plastic Petri dishes (Labserv, Auckland, New Zealand) in 20 ml DMEM+NCS. For static culture experiments the cells were plated into 24-well multi-dishes (NUNC A/S, Roskilde, Denmark) at a density of 0.5×10^6 live cells/ml per well. In both cases cells were incubated for 20 ± 2 h at 37 °C in a water-saturated, 5% CO₂:95% air atmosphere.

Multi-column perfusion experiments

The multi-column perfusion system, as described previously and with modifications (McIntosh & McIntosh 1983, Evans *et al.* 1985, 1988, 1996), allows for the simultaneous perfusion of up to 15 cell chambers, or columns. Through the use of a solenoid switching system various trains of peptide hormones and pharmacological agents can be applied to the cells.

Following overnight incubation the cells from each culture plate were recovered by centrifugation and the supernatant was replaced with 170 µl of a slurry of pre-swollen Sephadex G-25 fine (Sigma) suspended at a ratio of 1:1.5 (v/v) in Krebs-Ringer solution (KR; 125 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 3.6 mM NaHCO₃, 25 mM Hepes, 10 mM glucose and antibiotic/antimycotic agents as above). Slurries of 80 µl Bio-Gel P2 (Biorad Laboratories, Hercules, CA, USA) suspended 1:1.5 (v/v) in KR) and 80 µl Sephadex G-25 fine suspended 1:1.5 (v/v) in KR were added sequentially to the columns to form a bed on which the mixture of cells and Sephadex was placed. Perfusion was commenced at a flow rate of 0.16 ml/min with the perfusion medium (KR containing 0.05% alkali-treated casein (Livesey & Donald 1982) and 0.005% ascorbate (Sigma); KR/ATC) and the cells being maintained at 37 °C by a water jacket surrounding the columns and the media reservoirs. Effluent from the columns was collected in 5 and 10 min fractions and frozen for subsequent measurement of ACTH concentration by RIA.

During an experiment cells were perfused with a 'basal' solution (i.e. KR/ATC) and a 'test' solution containing either AVP or CRH. The tubing carrying these solutions was connected to the pump tubing by a Y-junction. At any given time one of these lines was clamped closed. Through the use of a solenoid-actuated switching system the perfusion solution could be rapidly and precisely changed between the 'test' and 'basal' solutions. When the cells were perfused with 'basal' KR/ATC plus two separate 'test' solutions, the basal line was manually transferred between KR/ATC and the second 'test' solution.

A variety of different experimental protocols were used in perfusion experiments. While the protocol used depended on the aim of the particular experiment, the design of all experiments shared some common features. These are described below while the details of the specific treatment regimes used in individual experiments can be found in Results. Each of the 15 columns in the perfusion system was randomly assigned an experimental treatment. Typically, each of the different treatments was repeated in at least two independent columns. In all experiments cells were perfused with 'basal' KR/ATC for at least 90 min at the beginning of the experiment, allowing them to recover from transfer into the columns. During this period ACTH secretion dropped to a consistently low level.

Experimental treatments began at the end of this run-in period.

Static culture experiments

Following overnight incubation the culture medium was removed from each well and replaced with 1 ml KR/ATC. After a 60 min pre-incubation at 37 °C this buffer was replaced with 1 ml KR/ATC containing CRH at various concentrations. The cells were treated with CRH for a range of times and then washed once with KR/ATC. One millilitre of KR/ATC, with or without CRH, was then added to each well to start the 90 min test incubation, which was carried out at 37 °C. At the completion of the test incubation, a sample of buffer was removed and frozen for subsequent analysis of ACTH concentration by RIA.

RIA

ACTH RIA was performed as previously described (Evans *et al.* 1985). The antiserum used (rabbit anti-porcine ACTH) was a gift from Dr Richard Donald, Christchurch Hospital (Christchurch, New Zealand) and the highly purified ovine ACTH, used for ¹²⁵I-radioiodination and assay standards, was a gift from Dr C H Li, Hormone Research Laboratory, University of California (San Francisco, CA, USA). Intra- and inter-assay coefficients of variation were 5.71 and 6.14% for static culture samples and 6.69 and 9.46% for multi-column perfusion samples.

Data and statistical analysis

In perfusion experiments the ability of a particular treatment to induce desensitization was assessed by measuring its effect on the response to a subsequent 5 min stimulation with either 100 nM AVP or 10 nM CRH. The magnitude of the response to each of these 'test' pulses was calculated as the total amount of ACTH released in the period following commencement of the pulse (20 min for AVP or 30 min for CRH), minus basal secretion (calculated using the mean value of the hormone concentration in the two or three fractions immediately preceding either the pulse or the pretreatment). Therefore the term 'response' refers to stimulated ACTH secretion. For the purposes of analysis the results from each perfusion column were treated as *n*=1. In general, for each treatment there were two or three replicates (columns) per experiment (one cell preparation) and there were two or three experiments. Data are reported as means ± S.E.M.

Static culture experiments were repeated three or four times using different cell preparations. Within each experiment triplicate or quadruplicate observations were made for each treatment. The data are reported as means ± S.E.M. with the *n* value equal to the number of experiments. Stimulated ACTH secretion was calculated by subtracting the unstimulated ACTH release (i.e. ACTH release in

the absence of stimulating agent) from the total ACTH release occurring in the presence of CRH during the test incubation.

Statistical significance was assessed using either Student's *t*-test or ANOVA as indicated in the text. $P < 0.05$ was considered significant.

Results

Desensitization of the ACTH response to AVP

The first series of experiments was designed to determine the concentrations and durations of AVP treatment which cause desensitization. The protocol for these experiments was based on that used by Weiss *et al.* (1995) to investigate desensitization of gonadotrophin-releasing hormone (GnRH)-stimulated luteinizing hormone secretion. In this procedure perfused cells are repeatedly stimulated with short pulses of a secretagogue at a high concentration. The interval between each of these pulses is relatively long to minimize the effects of one pulse on the next. In order to induce desensitization one of these pulses is immediately preceded by a treatment with a lower concentration of the secretagogue. The extent of desensitization is assessed quantitatively for each perfusion column by expressing the response to this pretreated pulse as a percentage of the mean of the responses to the other pulses, which act as controls.

Using the multi-column perfusion system, dispersed ovine anterior pituitary cells were stimulated with 5 min pulses of 100 nM AVP after 100, 180 and 260 min of perfusion. Responses to consecutive AVP pulses had a tendency to decrease. Compared with the first pulse the responses to the second and third pulses were $99.1 \pm 3.2\%$ ($n=3$, not significant (NS), one-way ANOVA with Dunnett's test) and $80.3 \pm 5.5\%$ ($n=3$, NS, one-way ANOVA with Dunnett's test) respectively (Fig. 1A). Since the mean of the responses to the first and third pulses was no different from the response to the second pulse ($n=9$, NS, Student's *t*-test) it was assigned as control (100%). Pretreatment for 25 min with 5 nM AVP prior to the second 100 nM AVP pulse (Fig. 1B) reduced the response to $62.1 \pm 3.7\%$ ($n=7$, $P < 0.0001$, *t*-test) of control. This pretreatment had no effect on the response to the third 100 nM AVP pulse when this was compared with the corresponding pulse in columns where there was no AVP pretreatment ($n=3$, NS, *t*-test).

The reduction in response to AVP following pretreatment with AVP seen in Fig. 1B reflected a specific desensitization of the cells to AVP and was not due to depletion of ACTH stores. This was demonstrated by replacing the three AVP pulses with 5 min pulses of 50 mM KCl. As shown in Fig. 2A there was no significant difference between the magnitude of the response to three consecutive pulses of 50 mM KCl: the responses to the second and third pulses were $93.3 \pm 3.5\%$ ($n=3$, NS,

Figure 1 Desensitization of the ACTH response to AVP. Representative data are shown from two independent columns of the perfusion system illustrating the protocol used in these experiments. (A) Cells were treated with 5 min pulses of 100 nM AVP after 100, 180 and 260 min of perfusion (indicated by arrows). (B) To induce desensitization the second pulse was preceded by a pretreatment with 5 nM AVP for 25 min (indicated by the black bar).

one-way ANOVA with Dunnett's test) and $83.9 \pm 4.5\%$ ($n=3$, NS, one-way ANOVA with Dunnett's test) of the response to the first pulse. However, pretreatment with 5 nM AVP for 25 min prior to the second pulse did not cause any reduction in response to the second KCl pulse compared with the control (Fig. 2B). In fact, there was a significant increase in ACTH secretion in response to the KCl pulse following AVP pretreatment (ACTH secretion increased to $198.8 \pm 18.4\%$ compared with control, $n=3$, $P < 0.05$, *t*-test). This is consistent with a report by Le Beau & Mason (1998) who found that AVP and KCl were synergistic at low concentrations. Overall, these results clearly show that the reduction in response to an AVP pulse following pretreatment is the result of an AVP-specific desensitization process.

Effect of concentration of AVP pretreatment on desensitization

To investigate the concentration-dependency of desensitization, the duration of the pretreatment was held

Figure 2 Effect of treatment with KCl on ACTH secretion. (A) Cells were treated with 5 min pulses of 50 mM KCl after 100, 180 and 260 min of perfusion (indicated by arrows). (B) Pretreatment of the second KCl pulse with 5 nM AVP for 25 min (indicated by black bar) resulted in an increase in ACTH secretion compared with the control pulses.

constant at 25 min while the AVP pretreatment concentration applied to different columns was varied from 0.1 to 50 nM. AVP pretreatment desensitized the response to a subsequent AVP pulse in a concentration-dependent manner. A sigmoidal concentration-response curve was fitted to the data yielding an r^2 of 0.93 and a predicted value for 50% desensitization (IC_{50}) of the ACTH response of 6.54 nM AVP. The lowest concentration of AVP tested capable of causing a significant reduction in response was 2.0 nM ($n=3$, $P<0.02$, t -test). Results are summarized in Fig. 3. ACTH release following pretreatment with 50 nM AVP was maintained at a plateau by the second AVP pulse rather than the pulse causing a response peak as seen at lower pretreatment concentrations.

Effect of duration of AVP pretreatment on desensitization

The degree of desensitization of the response to an AVP pulse was also found to be dependent on the duration of the pretreatment. A similar protocol to that described above was used except that the concentration of the

Figure 3 Concentration-dependency of the desensitization of the ACTH response to AVP, determined using the experimental protocol shown in Fig. 1. The ACTH response to the second AVP pulse was expressed as a percentage of the control pulses. The concentrations used for the 25 min pretreatment are plotted on a log scale. Data are means $\pm$ S.E.M. ($n=2-9$ for each treatment).

pretreatment was held constant at 5 nM AVP while the duration varied from 0 to 25 min. A pretreatment for as little as 5 min resulted in a significant reduction in the response to the second AVP pulse compared with the control (to $78.5 \pm 1.5\%$, $n=3$, $P<0.005$, t -test). A greater reduction in the response to the second AVP pulse (to $60.6 \pm 8.6\%$, $n=3$, $P<0.05$, t -test) was observed when the duration of the 5 nM AVP pretreatment was increased to 10 min. However, extending the pretreatment period beyond 10 min did not result in any further increase in the magnitude of desensitization. Data are summarized in Fig. 4.

Resensitization of the ACTH response to AVP

Perfused anterior pituitary cells were clearly quite sensitive to desensitization of the ACTH response to AVP. To characterize this process further, the ability of desensitized cells to recover their responsiveness to AVP was investigated. Since it was anticipated that the effects of desensitization would take somewhat longer to be reversed than they did to occur, an experimental protocol different from that used to investigate the time- and concentration-dependency of desensitization was employed. Cells were stimulated with a single 5 min pulse of 100 nM AVP after 200 min of perfusion. This pulse was preceded by a 15 min pretreatment with 10 nM AVP. Unlike the experimental protocol used in the first series of experiments the cells were allowed a recovery period of up to 80 min between the pretreatment and the AVP pulse. During this recovery period they were perfused with buffer alone. In order to assess quantitatively the extent of resensitization, the response of the pretreated cells to 100 nM AVP was expressed as a percentage of the mean

Figure 4 Time-dependency of the desensitization of the ACTH response to AVP. The ACTH response to the second AVP pulse, as a percentage of the control pulses, is plotted against the duration of the AVP pretreatment. The concentration of AVP used for the pretreatment was 5 nM. Data are means $\pm$ S.E.M. ($n=3-7$ for each treatment).

response of control cells (i.e. cells that had not been pretreated) to a 100 nM AVP pulse.

When the AVP pulse was applied immediately upon termination of the pretreatment (i.e. 0 min recovery time) the magnitude of the response to the test pulse was reduced to $36.7 \pm 5.4\%$ ($n=7$, $P<0.01$, one-way ANOVA with Dunnett's test) of controls that were not pretreated. When the cells were allowed a 10 min recovery period between the pretreatment and the test pulse there was a partial recovery in response to $67.3 \pm 7.6\%$ ($n=8$, $P<0.01$, Dunnett's test) of control. Recovery was complete after 40 min. Data are summarized in Fig. 5.

Desensitization of the ACTH response to CRH

Kinetic analysis of data obtained with perfused rat (Watanabe & Orth 1987, Won *et al.* 1990) and sheep (Mason 1988, Mason *et al.* 1998) anterior pituitary cells has shown that AVP-stimulated ACTH secretion peaks rapidly and then falls almost as rapidly to a low ($\sim 25\%$ of

Figure 5 Time-course of resensitization of the ACTH response to AVP. The ACTH response after pretreatment with 10 nM AVP for 15 min is plotted against the duration of the recovery period (i.e. the period of time between the pretreatment and the AVP pulse during which the cells were perfused with medium alone). Data are means $\pm$ S.E.M. ($n=7-15$ for each treatment).

peak), sustained plateau, despite continued AVP perfusion (e.g. see the response to the AVP pretreatment in Fig. 1B). In contrast, CRH-stimulated ACTH release reaches a maximum somewhat less rapidly and then remains constant, or declines very slowly, throughout the CRH exposure (e.g. see the response to the CRH pretreatment in Fig. 7C). These differences in the ACTH secretion profiles may indicate a slower and/or lower magnitude of desensitization for CRH than AVP. In view of the possibility that desensitization of the ACTH response to CRH is a relatively slow process, initial experiments were done using a static culture system. Cells were exposed to 1 nM CRH during a 90 min test incubation following pretreatment in the absence or presence of CRH (0.001–100 nM) for times ranging from 30 to 360 min. CRH pretreatment resulted in a concentration- and duration-dependent reduction in the ACTH response to subsequent stimulation with CRH (Fig. 6A and B). For example, with a 60 min pretreatment (Fig. 6A) the lowest concentration of CRH tested that was capable of causing a significant reduction in response was 0.1 nM ($n=4$, $P<0.05$, t -test). When a pretreatment CRH concentration of 10 nM was used (Fig. 6B) 60 min was the minimum duration capable of causing a significant reduction in response ($n=4$, $P<0.05$, t -test).

Perfusion was used in subsequent experiments to define more precisely the threshold concentration and duration required for CRH desensitization, and to enable easier comparison of the characteristics of AVP and CRH

Figure 6 Desensitization of the ACTH response to CRH in static culture. (A) The effect of a 60 min pretreatment with CRH, at the concentrations shown, on the ACTH response to a subsequent 90 min test incubation with 1 nM CRH. (B) The effect of pretreatment with 10 nM CRH for the indicated times on the response to a subsequent 1 nM CRH stimulation. Data are means $\pm$ S.E.M. ($n=3-4$ for each treatment).

desensitizations. Because of the relative slowness of the CRH desensitization seen in static culture, the 'three pulse' protocol used to investigate AVP desensitization was not used for CRH. Instead cells were stimulated with a single pulse of 10 nM CRH after 200 min of perfusion. This pulse was immediately preceded by CRH treatments of either 25 or 50 min duration and 0.1 or 1 nM concentration. The effect of these pretreatments on the response to the pulse was assessed by expressing the response to the CRH pulse as a percentage of the mean response of control cells (i.e. cells that had not been pretreated) to a 10 nM CRH pulse. In the control columns treatment with the 10 nM CRH pulse caused a broad peak of ACTH secretion (Fig. 7A). In test columns cells were pretreated with 0.1 or 1.0 nM CRH for either 25 or 50 min immediately prior to the CRH pulse (Fig. 7B and C). Analysis of the data by two-way ANOVA showed that both the concentration ($P<0.01$) and duration ($P<0.001$) of pretreatment were significant factors affecting the extent of desensitization observed. Further analysis showed that neither of the two 0.1 nM CRH pretreatments was capable of causing a significant reduction in the ACTH response to a subsequent CRH pulse (NS, two-way ANOVA with Dunnett's test; $n=9$ and $n=8$ respectively for the 25 and 50 min pretreatments). Significant decreases in the response to the test pulse were observed when the CRH pretreatment concentration was increased to 1 nM: compared with controls that were not pretreated the response to the pulse was reduced to $71.8 \pm 6.5\%$ ($n=9$, $P<0.05$, Dunnett's test) of control after a 25 min pretreatment and to $52.4 \pm 7.5\%$ ($n=9$, $P<0.01$, Dunnett's test) after a 50 min pretreatment. Results are summarized in Fig. 8.

Resensitization of the ACTH response to CRH

The time required for resensitization of the ACTH response to CRH to occur was investigated in a manner

similar to that used to investigate resensitization to AVP. Desensitization was induced by treatment with 1 nM CRH for 50 min. A recovery period of up to 100 min (during which cells were treated with basal KR) was allowed between the end of this pretreatment and the start of a 5 min CRH (10 nM) test pulse. When the pretreatment immediately preceded the pulse the response was reduced to $54.0 \pm 6.2\%$ ($n=6$, $P<0.01$, Dunnett's test) of control. Increasing the duration of the recovery period resulted in a progressive increase in ACTH responsiveness to the CRH pulse, returning to control levels after a 100 min recovery period. Results are summarized in Fig. 9.

Effect of CRH treatment on desensitization of the ACTH response to AVP

CRH and AVP both contribute to the regulation of ACTH secretion *in vivo*. Since it has been suggested that CRH may function as a permissive signal, setting the overall responsiveness of the corticotroph, while AVP is the main dynamic hypothalamic ACTH-releasing factor (Antoni 1993, Evans *et al.* 1996) we wished to determine whether a constant perfusion with CRH could overcome or enhance the desensitizing effects of AVP. Desensitization of the ACTH response to AVP was induced and assessed as shown in Fig. 1A and B, with the second of the three 100 nM AVP pulses being immediately preceded by a 15 min pretreatment with 5 nM AVP. As expected, this pretreatment markedly reduced the response (to $66.3 \pm 3.7\%$, $n=10$, $P<0.0001$, one-way ANOVA with Tukey's test). To investigate the effect of CRH on this AVP-induced desensitization, cells were continuously perfused with a low level of CRH (0.01 nM) beginning at 80 min and continuing until the end of the experiment. Although this concentration of

Figure 7 Desensitization of the ACTH response to CRH in multi-column perfusion. Representative data are shown from three independent columns of the perfusion system illustrating the protocol used in these experiments. (A) Cells were treated with a single 5 min pulse of 10 nM CRH after 200 min of perfusion (indicated by arrows). To induce desensitization this pulse was immediately preceded by a pretreatment with a lower concentration of CRH. (B) Twenty-five minute pretreatment with 0.1 nM CRH (open bar). (C) Fifty minute pretreatment with 1 nM CRH (black bar).

CRH was insufficient on its own to stimulate ACTH release, it was able to synergize with AVP causing enhanced ACTH secretion. Despite this effect on ACTH release, constant CRH perfusion did not overcome or enhance AVP-induced desensitization: in the presence of CRH, pretreatment with 5 nM AVP for 15 min reduced the response to $62.0 \pm 1.9\%$ ($n=13$, $P<0.0001$, Tukey's test) of controls that were not pretreated, but the magni-

Figure 8 Concentration- and time-dependency of desensitization of the ACTH response to CRH in multi-column perfusion. The ACTH response to CRH following pretreatment with CRH at the concentrations and durations indicated is shown. Data are means $\pm$ S.E.M. ($n=8-9$ for each treatment). Asterisks indicate statistically significant differences between the pretreated and control values (* $P<0.05$; ** $P<0.01$).

tude of this desensitization was not significantly different from that observed in the absence of CRH (difference 4.3%, NS, Tukey's test). Results are summarized in Fig. 10.

Discussion

This study has shown that the ACTH response of cultured ovine anterior pituitary cells undergoes rapid desensitization during treatment with CRH and AVP. The characteristics of the two desensitization processes differ suggesting that they contribute differently to the physiological regulation of ACTH secretion.

Earlier *in vitro* studies reported desensitization to AVP following stimulation with AVP at either high concentrations or for long periods of time (Holmes *et al.* 1984, Antoni *et al.* 1985, Evans *et al.* 1988, Castro 1993). In contrast, secretion of AVP into the hypophyseal portal circulation is highly pulsatile, with the AVP concentration of pulses normally being below 6 nM and secretory events lasting less than 30 min (Caraty *et al.* 1988, 1990, Engler *et al.* 1989, Battaglia *et al.* 1998, Dadoun *et al.* 1998). In this study desensitization of the ACTH response to AVP was found to occur following AVP treatment at concentrations and durations that more closely match those of

Figure 9 Time-course of resensitization of the ACTH response to CRH. The ACTH response after pretreatment with 1 nM CRH for 50 min is plotted against the duration of the recovery period (i.e. the period of time between the pretreatment and the CRH pulse during which the cells were perfused with medium alone). Data are means $\pm$ S.E.M. ($n=2-6$ for each treatment).

endogenous AVP pulses. Pretreatment with AVP at concentrations as low as 2 nM was capable of eliciting a significant reduction in response to a subsequent 100 nM AVP pulse ($IC_{50}=6.54$ nM) and the maximal desensitization to 5 nM AVP was reached with a 10 min pretreatment. The response returned to a level not significantly different from that of the controls after a recovery period of 40 min.

The comparison of our results with these endogenous AVP pulse characteristics is interesting. Our data suggest that while most endogenous AVP pulses would be long enough to cause desensitization, because of their low concentrations this would only be partial. This suggests that the role of desensitization is not to prevent ACTH secretion, but rather to limit the maximum secretory response to AVP. If this were the case, it would suggest that corticotrophs have an intrinsic set-point, beyond which they become refractory to further stimulation with AVP. Furthermore, the rapidity of the desensitization of the ACTH response to AVP might act to limit the duration of secretory episodes. Together these two properties of the desensitization process could result in a stereotyping of ACTH pulse amplitude and duration in response to AVP stimulation (Leng & Brown 1997). Weiss *et al.* (1995) used a similar experimental design to investigate the desensitization of perfused rat anterior pituitary cells to GnRH and found a qualitatively similar relationship between *in vitro* desensitization and endogenous pulse characteristics: endogenous GnRH pulses

Figure 10 Effect of CRH on desensitization of the ACTH response to AVP. Data show the ACTH response to an AVP pulse in either the presence or absence of a 0.01 nM CRH 'background' when the pulse was not preceded by a pretreatment (controls) and when it was immediately preceded by a 15 min pretreatment with 5 nM AVP. Data are means $\pm$ S.E.M. ($n=6-10$ for each treatment). Asterisks indicate statistically significant differences between the pretreated and control values (***) ($P<0.001$).

would have been long enough although not of sufficient concentration to evoke desensitization.

The ACTH response of sheep anterior pituitary cells to CRH appears to be considerably less susceptible to desensitization than the response to AVP. In perfusion experiments, significant desensitization following pretreatments of 25 or 50 min duration required a CRH concentration of >0.1 nM. Similar results were obtained in static culture experiments. We have observed with ovine anterior pituitary cells that CRH is a more potent stimulator of ACTH secretion than AVP; for CRH the threshold and maximal concentrations were 0.01 and ≥ 1 nM respectively, compared with values of 1 and ≥ 100 nM for AVP (AJ Johnson & DR Mason, unpublished observations). Thus the ACTH response to CRH was relatively less susceptible to homologous desensitization than the response to AVP. This difference in the regulation of the responses to the two hormones suggests that they might have distinct physiological roles in the regulation of ACTH secretion and are probably mediated via distinct molecular processes.

In contrast to AVP, the CRH concentrations and durations at which desensitization occurred were not within the endogenous ranges. In the sheep, CRH pulses during acute stress rarely last more than 30 min, with maximal portal plasma concentrations being less than ~ 0.4 nM (Caraty *et al.* 1988, 1990). Our data are inconsistent with evidence provided by Evans *et al.* (1988) indicating that the minimal concentration required for CRH-induced desensitization to repetitive 10 min CRH pulses in sheep anterior pituitary cells is 0.2 nM. This concentration corresponds with the peak CRH level seen in portal blood during acute haemorrhage, and is less than the level seen with insulin-induced hypoglycaemia. The differences between the results of the two studies may be a reflection of the different protocols used to examine CRH-induced desensitization. In any event, endogenous CRH pulses are unlikely to cause more than minimal desensitization during acute stress. However, if pituitary portal CRH concentrations are elevated during chronic stress or other pathophysiological conditions this could result in desensitization of pituitary ACTH responses to this hormone.

In view of the proposal that AVP is the primary acute signal for ACTH release and CRH acts to set the gain of corticotroph responsiveness to fluctuations in AVP (but is not a major regulator of short term fluctuations in ACTH secretion) (Antoni 1993, Evans *et al.* 1996), we examined the effect of CRH on AVP-induced desensitization. We found that the presence of CRH did not affect the extent of desensitization of the response to the second AVP pulse, which was reduced by $\sim 40\%$ following AVP pretreatment. Thus a low-level 'background' of CRH neither protected the ACTH response from AVP-induced desensitization nor enhanced the desensitization process. However, given the synergistic effect of CRH on AVP-stimulated ACTH secretion it seems certain that any desensitization of the response of corticotrophs to CRH could have significant consequences for AVP-induced ACTH secretion and thus stress-induced ACTH secretion.

The characteristics of the desensitization of the ACTH response to CRH and AVP give some clues to the mechanisms that underlie it. Both desensitizations occurred within minutes of stimulation and were readily reversible. These characteristics are typical of desensitization processes mediated by receptor uncoupling (Lohse 1993). Commonly uncoupling is mediated by receptor phosphorylation which, either directly or indirectly, prevents receptors from interacting with their associated G proteins (Lohse 1993). It has recently been reported that G protein-coupled receptor kinase (GRK) 3 is involved in desensitization of human CRH receptors of the sub-type found in the pituitary (i.e. CRH-R1) (Dautzenberg *et al.* 2001). It is possible that similar mechanisms are involved in regulating ovine pituitary CRH-R1. Phosphorylation of the pituitary AVP (V1b) receptor has not been reported

to date although the receptor terminus contains one proximal GRK consensus motif and two centrally located protein kinase C consensus motifs indicating that desensitization could occur via phosphorylation. Involvement of other mechanisms, such as receptor sequestration, cannot be excluded. The rapid desensitization observed in our experiments is more likely to be involved in regulating the acute response to AVP than in altering the responsiveness of the corticotroph during chronic stress.

In summary, it has been shown that the ACTH response to both CRH and AVP undergoes desensitization. The concentrations and durations of AVP exposure which are capable of eliciting desensitization suggest that this desensitization process may play an important physiological role in regulating ACTH secretion from the anterior pituitary. The role of CRH desensitization in the regulation of the hypothalamic-pituitary-adrenal axis is less clear, but if, as has been suggested, CRH acts to set corticotroph gain, any change in responsiveness to this neuropeptide might play an important role in the physiological control of ACTH secretion.

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