

Effects of Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) and Vasoactive Intestinal Polypeptide (VIP) on Hormone Secretion from Sheep Pituitary Cells *in vitro*

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Abstract

Although vasoactive intestinal polypeptide (VIP) is thought to be a prolactin releasing factor, *in vivo* studies on sheep suggest that it is inactive in this species. Recent studies, based primarily on the rat, suggest that the related pituitary adenylate cyclase-activating polypeptide (PACAP) is also a hypophysiotrophic factor but again in sheep, this peptide has no *in vivo* effects on hormone secretion despite being a potent activator of adenylate cyclase *in vitro*. This lack of response to either peptide *in vivo* in sheep could be due to the low concentration of peptide that reaches the pituitary gland following peripheral injection. In the present study we therefore adopted an alternative approach of evaluating *in vitro* effects of these peptides on GH, FSH, LH or prolactin secretion from dispersed sheep pituitary cells. In a time-course study, PACAP (1 $\mu\text{mol/l}$) increased GH concentrations in the culture medium between 1 and 4 h and again at 12 h but had no effect in the 6 and 24 h incubations. Prolactin, LH and FSH were not affected by PACAP. The response to various concentrations of PACAP (1 nmol/l–1 $\mu\text{mol/l}$) were then evaluated using a 3 h incubation. Again prolactin and LH were not affected by PACAP and there was a small increase in GH concentrations but only at high concentrations of PACAP (0.1 and 1 $\mu\text{mol/l}$; $P < 0.05$). PACAP also stimulated FSH secretion in cells from some animals although this effect was small. The GH response to PACAP was inhibited by PACAP(6–38), a putative PACAP antagonist, but not by (*N*-Ac-Tyr¹, D-Arg²)-GHRH(1–29)-NH₂, a GH-releasing hormone (GHRH) antagonist. The cAMP antagonist Rp-cAMPS was unable to block the GH response to PACAP suggesting that cAMP does not mediate the secretory response to this peptide. At incubation times from 1–24 h, VIP (1 $\mu\text{mol/l}$) had no effects on prolactin, LH or GH secretion and, in a further experiment based on a 3 h incubation, concentrations of VIP from 1 nmol/l–1 $\mu\text{mol/l}$ were again without effect on prolactin concentrations. Interactions between PACAP and gonadotrophin releasing hormone (GnRH), GHRH and dopamine were also investigated. PACAP (1 nmol/l–1 $\mu\text{mol/l}$) did not affect the gonadotrophin or prolactin responses to GnRH or dopamine respectively. However, at a high concentration (1 $\mu\text{mol/l}$), PACAP inhibited the GH response to GHRH. In summary, these results show that PACAP causes a modest increase in FSH and GH secretion from sheep pituitary cells but only at concentrations of PACAP that are unlikely to be in the physiological range. The present study confirms that VIP is not a prolactin releasing factor in sheep.

Vasoactive intestinal polypeptide (VIP) and the more recently discovered pituitary adenylate cyclase-activating polypeptide (PACAP) are related peptides that are thought to have important roles in regulating pituitary function. At least for some species, there is now considerable evidence implicating VIP as a prolactin releasing factor. In the rat, VIP is found within the external zone of the median eminence (1, 2), it occurs at high concentrations in hypophysial portal blood (3) and it stimulates the release of prolactin both *in vivo* and *in vitro* (4–6). VIP receptors occur in the pituitary gland (7) and it is presumably via these receptors that VIP induces Ca²⁺ influx, and therefore prolactin secretion, via cAMP dependent and independent mechanisms (8, 9). Furthermore, immunoneutralization of VIP prevents the release of prolactin induced by ether stress and delays the hormone

release induced by the sucking stimulus (10), indicating that this peptide stimulates prolactin secretion under physiological conditions. Experiments on humans and non-mammalian species such as the chicken provide further support for the notion that VIP is a prolactin-releasing factor (11, 12). However, perhaps surprisingly, evidence is now accumulating to indicate that, at least in sheep, VIP is not involved in pituitary function. In this species, VIP is found within the external zone of the median eminence but is not secreted into hypophysial portal blood (13) and, at least *in vivo*, does not induce prolactin secretion (14, 15). In contrast to the rat, cAMP is not a second messenger for VIP in sheep pituitary cells (16) but whether it influences hormone secretion *in vitro*, is not known.

The evidence implicating PACAP as a hypophysiotrophic factor

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is less convincing than for VIP. PACAP containing fibres are found within the external (sheep, (17); human, (18)) or internal (rat; (19, 20)) zones of the median eminence and, at least for the rat, PACAP has been detected in hypophysial portal blood (21). PACAP was named because of its ability to stimulate cAMP accumulation in rat pituitary cells (22), a feature that has now been demonstrated for several species (sheep, (16); frog, (23)) and a number of other cell types e.g. (24, 25). In addition, other signal transduction pathways such as the inositol phosphate pathway are also used by this peptide (26). In contrast to the unambiguous effects of PACAP on second messengers in pituitary cells, effects on pituitary hormone secretion are less striking. In the rat, peripheral injection of relatively large concentrations of PACAP is needed to stimulate secretion of GH, LH, ACTH and prolactin (27, 28). Nevertheless, these *in vivo* responses are supported by most, but not all, of the *in vitro* studies on dispersed rat pituitary cells in which PACAP stimulates secretion of these hormones (22, 29, 30). However, as with the *in vivo* work, relatively large concentrations of PACAP give small responses in comparison with the known hypophysiotrophic factors. In contrast, peripheral injection of PACAP into sheep or humans does not affect pituitary hormone secretion but it is not clear whether the concentrations used in these studies would have achieved high concentrations of PACAP at the pituitary level (15, 31). Further, although it is known that PACAP activates adenylate cyclase in primary cultures of sheep pituitary cells (16), effects on hormone secretion have not been reported.

The aim of this study was therefore to determine whether PACAP or VIP could influence secretion of several anterior pituitary hormones from sheep pituitary cells maintained in static culture. In addition to direct effects on hormone secretion, PACAP or VIP could influence anterior pituitary function by interacting with the known hypophysiotrophic factors such as GH-releasing hormone (GHRH) or gonadotrophin releasing hormone (GnRH) or the prolactin release-inhibiting factor dopamine and so this was also tested.

Results

Time course and concentration-response experiments

In an initial experiment replicated on cells from 4 pituitary glands, the response to PACAP (0.1 $\mu\text{mol/l}$) was determined at incubation times of 0.5, 1, 2, 3, 4, 6, 12 or 24 h, with control and peptide treated incubations terminated at each time point. In the absence of PACAP, the concentration of prolactin, GH, LH, and FSH in the culture medium increased with time which indicates constitutive secretion of hormone from the respective pituitary cell type. In the presence of PACAP, GH concentrations were significantly ($P < 0.01$) increased between 0.5 h and 4 h (Fig. 1). Thereafter GH concentrations remained above control values, although this difference was only significant at the 12 h incubation ($P < 0.01$). For prolactin, LH and FSH, PACAP had no significant effects at any time point. In a similar experiment with VIP (0.1 $\mu\text{mol/l}$), cells from two pituitary glands were studied independently and in neither case did VIP treatment affect the concentration of GH, prolactin, LH or FSH in the culture medium.

Based on the above results, the response to a 3 h incubation with increasing concentrations of PACAP (1 nmol/l–1 $\mu\text{mol/l}$) was tested. The results in Fig. 2 are pooled from 6 separate

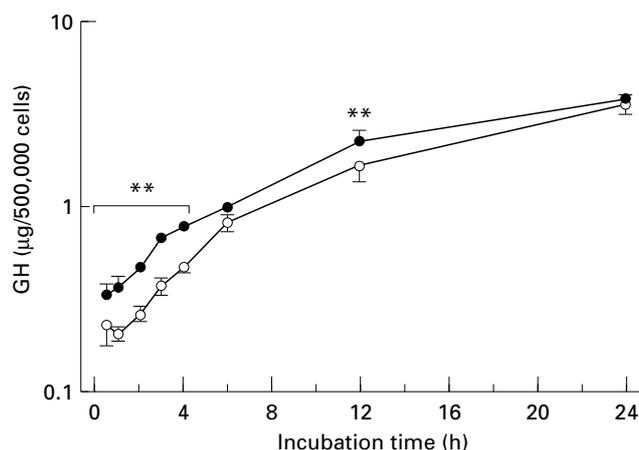


FIG. 1. Time course relationship for the effects of PACAP on secretion of GH from sheep pituitary cells. Cells were incubated with PACAP (0.1 $\mu\text{mol/l}$; ●) or incubation medium alone (○). Data are the mean \pm SEM from 4 separate experiments. ** $P < 0.01$ compared with control.

experiments. PACAP significantly increased GH concentrations ($P < 0.05$, ANOVA) although only for the two highest concentrations (0.1 and 1 $\mu\text{mol/l}$) was the response significantly different from the control ($P < 0.05$). PACAP also had a significant effect on FSH secretion ($P < 0.05$, ANOVA) but no single concentration tested significantly different from the control in the pair-wise comparisons (Fig. 2B). For both the GH and FSH results, there was a significant interaction ($P < 0.01$) between animal and concentration which indicates the pattern of response to PACAP varied between animals. In contrast to GH and FSH, the concentrations of LH and prolactin were not affected by PACAP (results not shown). Potential effects of VIP on prolactin secretion were re-evaluated with the 3 h incubations and at a range of concentrations of VIP (1 nmol/l–1 $\mu\text{mol/l}$) but irrespective of concentration, VIP had no effect on prolactin concentrations (results not shown).

In order to show that the cultured ovine pituitary cells would respond to proven hypophysiotrophic factors, a limited number of experiments were undertaken with GHRH, GnRH and thyrotrophin-releasing hormone (TRH). The lowest concentration of GHRH to significantly ($P < 0.01$) stimulate GH secretion was 10 pmol/l for which GH concentrations were $170 \pm 10\%$ of the vehicle treated control ($n = 6$). For GnRH, FSH and LH secretion were stimulated at concentrations greater than 100 pmol/l. At this concentration LH and FSH concentrations were 425 ± 88 ($n = 6$) and $161 \pm 49\%$ ($n = 4$) of the control. A complete concentration-response curve was not determined for TRH but at a concentration of 10 nmol/l, prolactin concentrations increased to 361 and 533% (means of 2 separate experiments) of control values.

Interaction between PACAP and hypophysiotrophic factors

To determine whether the GH response to GHRH was affected by PACAP, ovine GHRH (30 pmol/l) was incubated with or without increasing concentrations of PACAP (1 nmol/l–1 $\mu\text{mol/l}$) for 3 h. The results pooled from 4 separate experiments show that, as expected, GHRH (30 pmol/l) stimulated ($P < 0.01$) the basal secretion of GH to 194% of the control (Fig. 3). PACAP also stimulated GH secretion but only at high concentrations (0.1

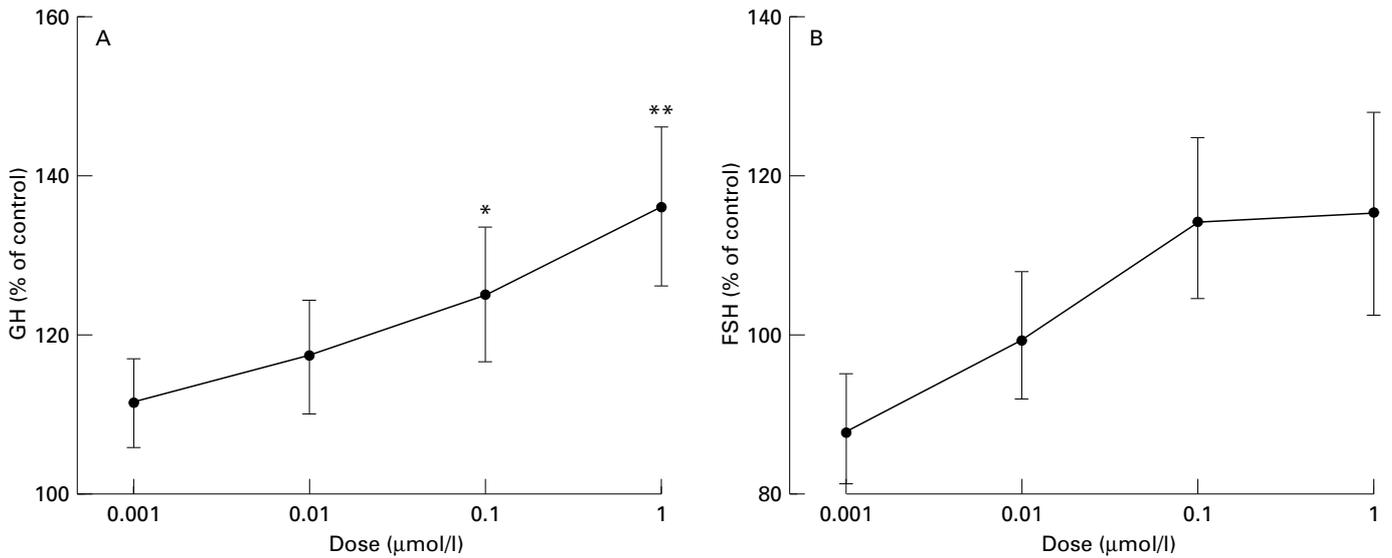


FIG. 2. Dose-response relationship for the effects of PACAP on secretion of (A) GH and (B) FSH from sheep pituitary cells. Cells were incubated with increasing concentrations of PACAP for 3 h. Values are the mean \pm SEM expressed as a percentage of their respective control from 6 separate experiments. * $P < 0.05$, ** $P < 0.01$ compared with control.

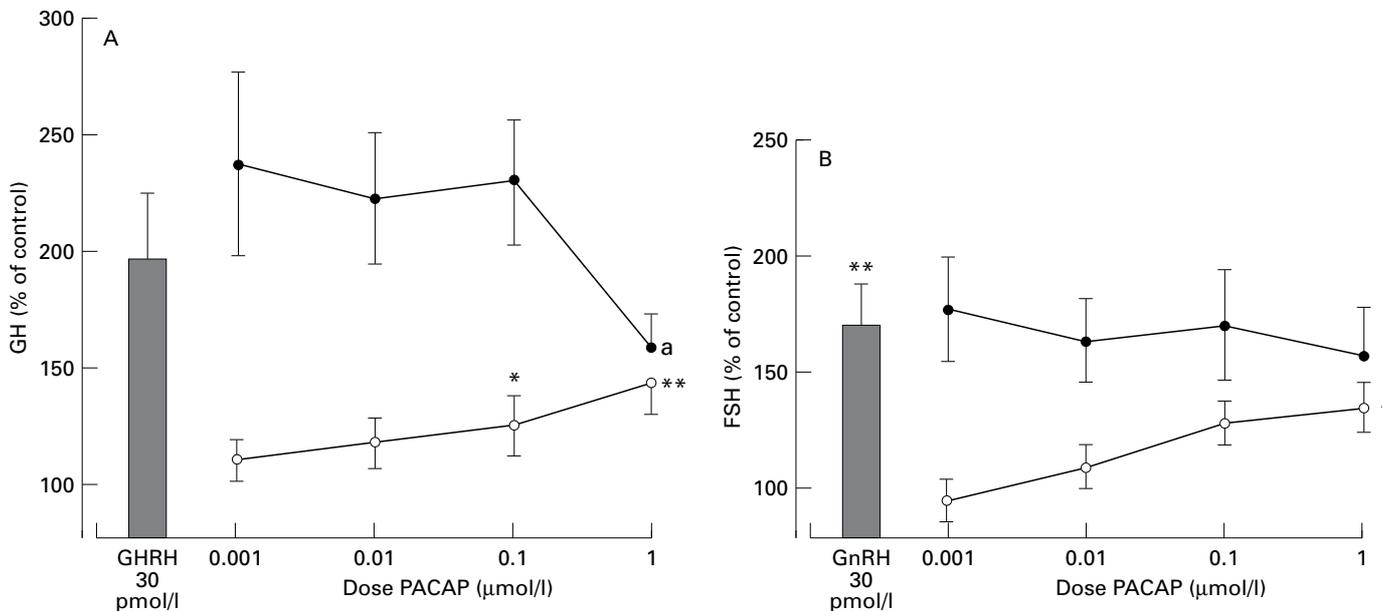


FIG. 3. Effect of coincubation of PACAP with (A) GHRH or (B) GnRH. Cells were incubated for 3 h with increasing concentrations of PACAP, with (○) or without (●) GHRH (30 pmol/l). Values are the mean \pm SEM from 4 separate experiments expressed as a percentage of their respective control. * $P < 0.05$, ** $P < 0.01$ compared with control; a, * $P < 0.05$ compared with GHRH alone.

and 1 µmol/l) was the effect significant ($P < 0.05$ and $P < 0.01$ respectively). When PACAP was coincubated with GHRH, the highest concentration of PACAP (1 µmol/l) significantly reduced the GH response to GHRH ($P < 0.05$). At lower concentrations, PACAP increased the response to GHRH but this effect was not significant.

A similar design was used to test for interactions between PACAP and GnRH (30 pmol/l). As expected, GnRH caused an increase in LH and FSH concentrations to 353 and 171% of the control respectively. In the absence of GnRH, PACAP had no

effect on LH secretion but caused a small increase in FSH secretion ($P < 0.05$, ANOVA) with the highest concentration (1 µmol/l) significantly increased over the control (Fig. 3). Co-incubation of PACAP with GnRH had no effect on either the LH or FSH responses to GnRH.

The next experiment was performed to investigate the effects of PACAP on prolactin secretion during a period of dopaminergic inhibition. Cells were incubated with PACAP (1 nmol/l–1 µmol/l) for 3 h in the presence or absence of dopamine (0.1 µmol/l) with the entire experiment replicated on 4 separate occasions.

Dopamine suppressed prolactin concentrations to 38% of the control and this effect was the same irrespective of whether PACAP was added to the incubation medium. In incubations receiving PACAP alone, mean prolactin concentrations increased with concentration but this effect was not significant (results not shown).

Antagonist experiments

The PACAP antagonist, PACAP(6–38), was tested for its ability to antagonize the GH response to PACAP. Cells were incubated for 3 h with increasing concentrations of PACAP (1 nmol/l–1 μ mol/l), with or without 1 μ mol/l PACAP(6–38). The results from one of two identical experiments in Fig. 4 show that, as expected, the 0.1 and 1 μ mol/l concentrations of PACAP significantly ($P < 0.05$) increased GH concentrations. PACAP antagonist (1 μ mol/l) alone had no effect on GH secretion but when coincubated with PACAP, it prevented the GH response to PACAP. When the experiment was repeated on cells from another animal, qualitatively similar results were obtained.

In an earlier experiment, PACAP (1 μ mol/l) inhibited the GH response to GHRH. However PACAP at the same concentration also stimulates GH secretion. It is possible therefore that PACAP may be a partial agonist at the GHRH receptor. In order to test this hypothesis (*N*-Ac-Tyr¹, D-Arg²)-GHRH(1–29)-NH₂, a GHRH antagonist, was used in an attempt to antagonize the response to PACAP. Cells were incubated with PACAP at various concentrations (1 nmol/l–1 μ mol/l) or the medium alone, with or without GHRH antagonist (10 nmol/l) for 3 h. To verify that the concentration of GHRH antagonist used in this experiment was effective as a GHRH antagonist, a parallel experiment was performed in which cells were incubated for 3 h with GHRH (30 pmol/l), with or without GHRH antagonist (10 nmol/l) or medium alone (control). The results in Fig. 5 show that the

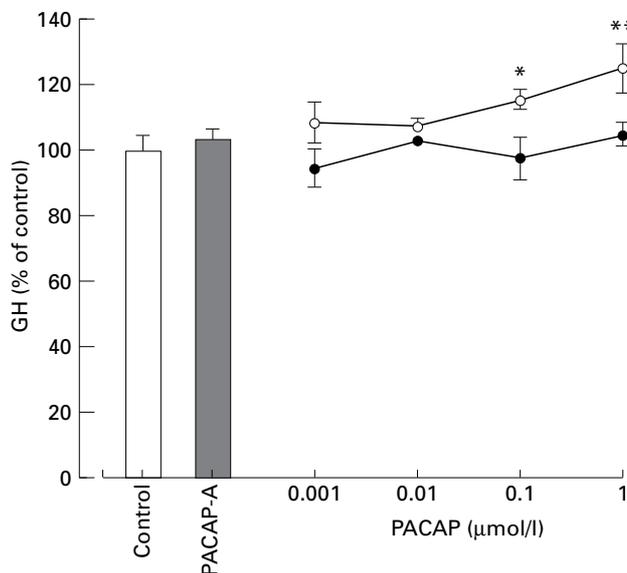


FIG. 4. Effects of a PACAP antagonist (PACAP-A), PACAP(6–38), on the GH response to PACAP. Cells were incubated for 3 h with increasing concentration of PACAP, with (○) or without (●) PACAP(6–38) (1 μ mol/l). Values are the mean \pm SEM of triplicate incubations expressed as a percentage of the control. * $P < 0.05$, ** $P < 0.01$ compared with control.

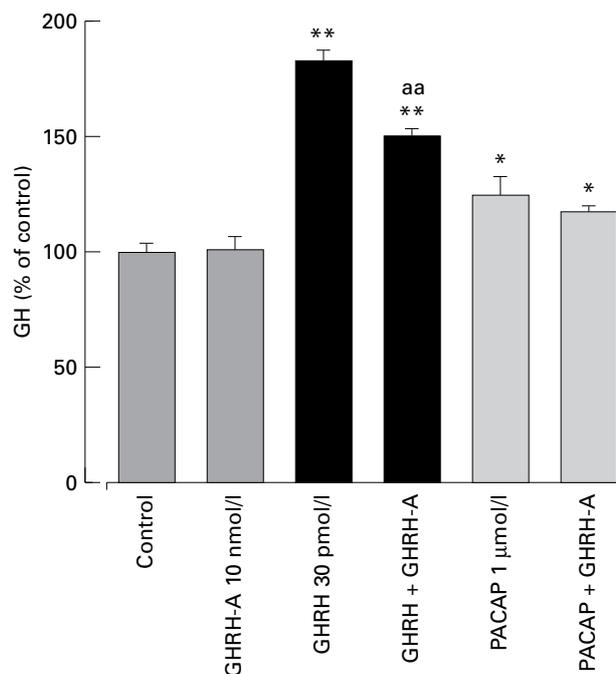


FIG. 5. Effects of (*N*-Ac-Tyr¹, D-Arg²)-GHRH(1–29)-NH₂, a GHRH antagonist (GHRH-A), on the GH response to GHRH and PACAP. Cells were incubated for 3 h with PACAP (1 μ mol/l) or GHRH (30 pmol/l), with or without GHRH-A (10 nmol/l). Values are the mean \pm SEM of triplicate incubations expressed as a percentage of the control. Data are representative of 2 experiments with similar results. * $P < 0.05$, ** $P < 0.01$ compared with control; aa, $P < 0.01$ GHRH + GHRH-A compared with GHRH alone.

GHRH antagonist had no effect on GH secretion. As expected, PACAP alone stimulated GH secretion with only the highest concentration (1 μ mol/l) significantly different ($P < 0.05$) from the control. Coincubation of GHRH antagonist with PACAP had no significant effect on the GH response to PACAP. GHRH alone stimulated GH secretion to approximately 170% of the control value ($P < 0.01$). This response was partially ($P < 0.05$ compared with GHRH) inhibited by the GHRH antagonist although GH concentrations were still elevated compared with the control ($P < 0.01$). When the experiment was repeated on cells from another animal, qualitatively similar results were obtained.

Finally, we wished to determine whether the GH response to PACAP was mediated by cAMP. Rp-cAMPS, a competitive antagonist of cAMP on cAMP-dependent protein kinase A (32, 33), was used to block cAMP-mediated effects subsequent to stimulation by PACAP. For comparison, the effect of Rp-cAMPS on the GH response to GHRH was also determined. Cells were preincubated with or without Rp-cAMPS (10 μ mol/l) for 30 min after which they were incubated with PACAP (1 μ mol/l) or GHRH (30 pmol/l), with or without Rp-cAMPS (10 μ mol/l) for 3 h. The results in Fig. 6 show that both GHRH and PACAP significantly increased ($P < 0.01$) GH concentrations and Rp-cAMPS had no effect by itself. However, in the presence of Rp-cAMPS, the GH response to GHRH was reduced ($P < 0.05$, compared to GH response to GHRH) but still remained significantly increased above control values ($P < 0.05$). In contrast, Rp-cAMP had no effect on the GH response to PACAP. Similar

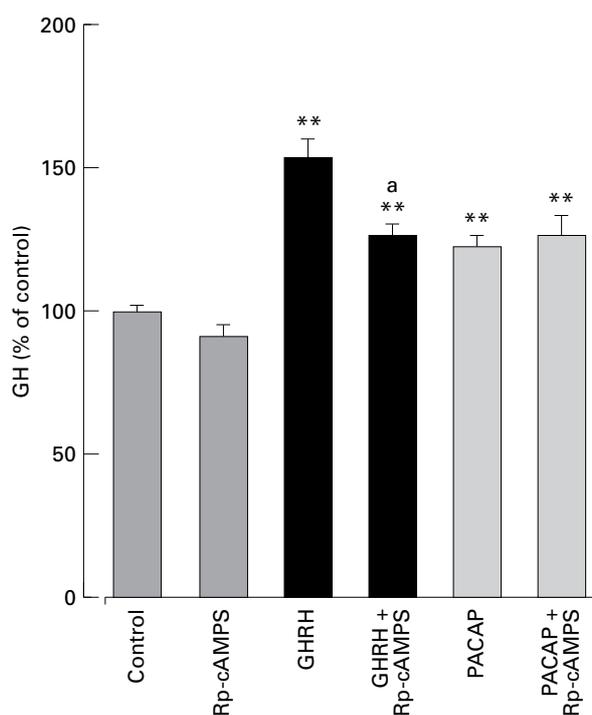


FIG. 6. Effects of adenosine 3',5'-cyclic monophosphothioate, Rp-isomer (Rp-cAMPS) on the GH response to GHRH or PACAP. Cells were incubated for 3 h with GHRH (30 pmol/l) or PACAP (1 μ mol/l), with or without Rp-cAMPS (10 μ mol/l). Values are the mean \pm SEM of triplicate incubations expressed as a percentage of the control. Data are representative of 2 experiments with similar results. ** $P < 0.01$ GHRH or PACAP vs control; a, $P < 0.05$ GHRH + Rp-cAMPS vs GHRH.

results were obtained when the experiment was repeated on cells from a second ewe.

Discussion

In this study, the effects of PACAP and VIP on GH, prolactin, LH and FSH secretion from sheep pituitary cells in primary culture were examined. The only significant response to be observed in the time-course studies was an increase in GH concentrations induced by PACAP between 1 and 4 h of incubation and again at 12 h. In further experiments based on a 3 h incubation, it was shown that only high concentrations of PACAP (0.1 and 1 μ mol/l) gave a significant GH response. This experiment was repeated on 6 animals at different times of the year and, as shown by the significant interaction between concentration and animal in the ANOVA, the responsiveness to PACAP varied between animals. This suggests that factors such as variations in laboratory technique, genetic variation between individuals or physiological state (e.g. seasonal effects, nutrition etc) of the animal before euthanasia could influence the *in vitro* responsiveness of pituitary cells to PACAP. The lowest concentration of PACAP that stimulated GH concentrations was more than three orders of magnitude greater than that required by GHRH and even at these large concentrations of PACAP, the GH response was relatively small. Similar observations have been made in dispersed pituitary cells from rats (22, 29) and cattle (34) in which concentrations of PACAP above 1 nmol/l increased GH

secretion whereas GHRH was effective at much lower concentrations (0.1 pmol/l).

PACAP also affected FSH secretion in the PACAP concentration-response study although, as with GH, the response was small and only occurred at high concentrations in comparison with the FSH response to GnRH. There were also differences in responsiveness when the treatments were replicated on cells from different sheep. In the concentration-response study based on pituitaries from 6 ewes, the overall effect of PACAP in the ANOVA was significant but, based on the pairwise comparisons with the control, no concentration was significantly increased from the control values. Four of these sheep were also used in a later experiment and, when analysed separately for this study, the highest concentration of PACAP did indeed stimulate FSH secretion. In addition to those factors considered above for GH, the variation in responsiveness to PACAP observed here for FSH might also be due to differences in reproductive status of the animals used in the various replicates which were done at different times of year and without regard for stage of the oestrous cycle.

The effects of PACAP on GH and FSH secretion in the present study only occurred at very high concentrations which are unlikely to be in the physiological range. To date, PACAP has only been detected in hypophysial portal blood from the rat where it occurs in concentrations of 50–100 pmol/l (21). Clearly this would give anterior pituitary tissue concentrations much lower than those required to cause a response in the present *in vitro* study. Present evidence suggests that local synthesis of PACAP does not occur in the anterior pituitary gland (17) and so it is unlikely that pituitary cells would receive high concentrations of PACAP by paracrine or autocrine routes. PACAP has been localized in the posterior pituitary gland (17) and so it is possible that the anterior pituitary could receive high concentrations of PACAP via the short hypophysial portal veins. Nevertheless, it seems unlikely that such high concentrations of peptide as were required in the present *in vitro* study could be achieved via this route.

It is also possible that the high concentrations required to evoke hormone secretion in the present study could be due to features of the *in vitro* system. For example, desensitization to PACAP could reduce the responsiveness of cells maintained in static culture and indeed we have observed such an effect in terms of the cAMP response to PACAP (16). Alternatively, the GH response to PACAP may be indirect due to release of some paracrine or autocrine substances. Once secreted, these would be diluted by the incubation medium, thereby necessitating high concentrations of PACAP to induce effective concentrations. The secretion and effects of such substances have yet to be identified.

Both PACAP and GHRH stimulate intracellular cAMP production and, at least for GHRH, cAMP is known to be an important second messenger involved in the GH secretory response (35). However, the eventual stimulus for GH secretion from the somatotroph is an elevation in intracellular Ca^{2+} concentration due to influx of extracellular Ca^{2+} (36, 37). In rat somatotrophs, GHRH induces Ca^{2+} influx by several mechanisms. Binding of GHRH to its receptor is known to activate adenylate cyclase resulting in increased intracellular cAMP. cAMP then binds to and activates protein kinase A (PKA) which then phosphorylates calcium channels allowing them to open during depolarisation (38, 39). There are also mechanisms which link the activated GHRH receptor to Ca^{2+} influx and which are independent of cAMP. For example, GHRH also activates PKC

to stimulate GH secretion (40) and the GTP-binding protein Gs, which is stimulated through activation of GHRH receptors by GHRH, may open calcium channels by membrane-confined mechanism (39, 41). Much less is known about the mechanisms through which PACAP induces GH secretion. In rat somatotrophs, PACAP causes an influx of Ca^{2+} which can be blocked by Rp-cAMPS (42). These findings suggest that PACAP causes GH secretion by increasing intracellular cAMP and activating PKA. However, in the present study based on a mixed pituitary cell population from the ewe, Rp-cAMPS had no effect on the GH response to PACAP. As a positive control we showed that Rp-cAMPS partially inhibited GH secretion stimulated by GHRH. This result is consistent with the above information that GHRH uses the cAMP/PKA signal transduction system to stimulate GH secretion. Because GHRH also uses other signal transduction pathways, Rp-cAMPS is only partially effective in blocking the GH response. Our observation that the GH response to PACAP was not blocked by Rp-cAMPS suggests that, at least in the ewe, PACAP uses signal transduction pathways other than the cAMP/PKA system to stimulate GH secretion. Further support for this notion comes from the observation that the lowest concentration of PACAP able to stimulate cAMP accumulation in sheep pituitary cells is 2 orders of magnitude below that required to stimulate GH or FSH secretion (16).

Possible interactions between PACAP and GHRH, GnRH or dopamine were also investigated in this study. Clearly PACAP did not modify the prolactin response to dopamine or the LH response to GnRH. Although, PACAP stimulated a small increase in FSH secretion at the highest concentration tested, this effect was not additive or antagonistic to the GnRH effects on FSH secretion. The only marked interaction that was observed was the antagonistic effect of PACAP on GHRH induced GH secretion which has not been previously observed in any species. This inhibition which occurred consistently in 4 separate experiments but only at the highest concentration of PACAP ($1 \mu\text{mol/l}$), could occur by many mechanisms. First, PACAP may be a partial agonist at the GHRH receptor but with low affinity and so effectively acts as an antagonist when co-incubated at high concentrations with GHRH. PACAP, VIP and GHRH belong to a superfamily of structurally related peptides (22, 43) and evidence of cross-reaction between various ligands and receptors does exist (44). Binding of PACAP to GHRH receptors is therefore possible and so in the present study, a GHRH antagonist was tested for its ability to block the GH response to PACAP. The GHRH antagonist, at a concentration which inhibited the GH response to GHRH, did not affect the GH response to PACAP suggesting that PACAP does not stimulate GH secretion via the GHRH receptor which is in accord with similar experiments done on rat pituitary cells (45). It is obvious that a similar result at higher concentrations of the GHRH antagonist would be more convincing. However the GHRH antagonist is a partial agonist at higher concentrations and so such an experiment could not be undertaken. On the other hand, a PACAP antagonist was able to block the GH response to PACAP. These results suggest that the stimulatory effect of PACAP on GH secretion is unlikely to involve GHRH receptors but rather PACAP receptors. However, again, the results with the PACAP antagonist are not conclusive in that binding of this compound to other receptors has not been extensively characterized.

PACAP receptors have not been identified or characterized

from any ovine tissues but in rats, 3 groups of PACAP/VIP receptors (PVR) have now been identified and cloned and all 3 subtypes occur in the anterior pituitary gland (see (46) for a review). PVR 1 which is also known as the PACAP type-I receptor is characterized by high affinity for PACAP, much lower affinity for VIP and coupling with adenylate cyclase and phospholipase-C. PVR 2 and PVR 3 (also known as PACAP/VIP type II binding sites), have similar affinities for PACAP and VIP and couple with adenylate cyclase. It is generally thought that PACAP stimulates GH secretion from rat somatotrophs by acting on either PVR 2 or 3 subtypes coupled to adenylate cyclase (46). This has been proven for GH3 cells (47) but, to our knowledge, not for rat somatotrophs. In contrast in the ewe, we have shown here that VIP does not stimulate GH secretion and that cAMP is not involved in the GH response to PACAP. In an earlier study we also reported that PACAP but not VIP is linked with adenylate cyclase in this species. Assuming that sheep have similar PVR subtypes to the rat, these results suggest that PACAP influences GH secretion via a PVR 1 subtype. However, it is also possible that the hormone secretory responses observed in the present study could be due to cross-reaction between PACAP and some other type of receptor.

The failure of VIP to stimulate prolactin secretion in sheep pituitary cells is in direct contrast to studies in the rat, human and chicken (4, 11, 12) in which VIP is a prolactin-releasing factor. However the lack of effect of VIP on prolactin *in vitro* is consistent with *in vivo* studies of the ewe (14, 15) in which intracarotid or intravenous administration of VIP had no effect on plasma prolactin concentrations. In addition, it has been recently shown that VIP is not secreted into hypophysial portal blood (13) of the ewe. Taken together, these studies now provide convincing evidence that this peptide is not a prolactin releasing factor in sheep.

In conclusion, these experiments on dispersed sheep pituitary cells show that PACAP stimulates GH and FSH secretion but only at concentrations well above the expected physiological range. No evidence was obtained to suggest that PACAP may augment the secretory response to GnRH, GHRH or dopamine. Results from experiments with several antagonists along with the observation that VIP had no effect on GH secretion suggest that PACAP influences GH via a PVR 1 linked to a second messengers other than cAMP. These experiments also confirm that VIP is not a prolactin releasing factor in this species.

Materials and methods

Reagents

The synthetic peptides PACAP and VIP were purchased from the Peptide Institute Inc. (Osaka, Japan) and had identical amino acid sequences to the native ovine peptides. PACAP(6–38) and ovine GHRH were purchased from Peninsula (Belmont, CA, USA). GnRH and dopamine were purchased from Sigma Chemical Co. (Castle Hill, NSW, Australia). (*N*-Ac-Tyr¹, D-Arg²)-GHRH(1–29)-NH₂, a GHRH antagonist, was purchased from AUSPEP, Parkville, Victoria, Australia. Adenosine 3',5'-cyclic monophosphothioate, Rp isomer trimethylammonium salt (Rp-cAMPS) was purchased from Calbiochem, La Jolla, California, USA. The source of tissue culture reagents have been reported in detail elsewhere (16).

Cell dispersion and culture

Pituitary glands from mature ewes were dispersed by incubation with trypsin as previously described in detail (16). Each preparation of cells

came from a single pituitary gland and was used in one experiment. Dispersed cells were plated into 24-well plates at a density of 500,000 cells/well and were preincubated for 3 days in Fetal Calf Serum-supplemented Dulbecco's modification of Eagle's medium (DMEM) before use. On the day of the experiment, cells were washed twice with DPBS+, once with Bovine Serum Albumin-DMEM (BSA-DMEM) and then were preincubated in 1 ml BSA-DMEM for 30 min. The experiment was started by replacing the BSA-DMEM with 1 ml of the same solution, with or without the test substance, and the incubation was then continued for an appropriate period. The incubation medium was then removed from the culture dish and stored at -20°C until assay. All treatments were done in triplicate and each experiment was replicated at least once.

Radioimmunoassays

Prolactin, GH, LH and FSH concentrations were determined by RIA as previously described (15, 48). Assay sensitivity was 1.6 ng/ml for prolactin, 0.4 ng/ml for GH and 0.9 ng/ml for LH and 0.3 ng/ml for FSH. The intra- and inter-assay coefficients of variation were 7.2 and 16.3% for the prolactin assay, 8.3 and 15.7% for GH, 8.8 and 13.8% for LH and 13.2 and 12.1% for FSH.

Statistical analysis

Data were analysed by one or two way analysis of variance (ANOVA). In experiments replicated on 4 or 6 occasions, each time with pituitary cells from a different sheep, a randomized block design with replication within blocks was used for the analysis. Where results of the ANOVA were significant, Dunnett's test was used to compare treatment means with the control. Where required, a Student-Newman-Keuls test was used for comparisons between treatment means. For the time-course studies, there was clear evidence of heterogeneity of variance. These data were therefore log transformed before analysis. Data from the remaining experiments were analysed without transformation. In some experiments, results are presented as a percentage of the control response.

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