

A COMPLEX MECHANISM OF FACILITATION IN PITUITARY ACTH CELLS: RECENT SINGLE-CELL STUDIES

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Summary


The transfer of information by chemical signals during complex biological processes can, with increasing frequency, be described in terms of interacting signal pairs. External signalling is rarely monolithic; rather, signal pairs are utilized in processes such as hormone secretion, neurotransmission, cell growth and differentiation. The dualism of external signalling often results in the occurrence of synergy. One signal appears to turn the cell on or off, and its synergistic partner increases cell responsiveness, providing gain control of the cellular response. ACTH release provoked by certain stressors arises from a synergistic interaction between two hypothalamic hormones: corticotropin releasing hormone (CRH) and a modulator such as vasopressin (AVP). The pituitary ACTH cell has been used to unravel the intracellular messenger equivalents of an external signal interaction that generates synergy.

Research emphasizes the single cell approach. Direct measurements of intracellular free Ca^{2+} were performed using the Ca^{2+} -sensitive fluorescent probe fura-2/AM and instrumentation for digital image processing. A reverse haemolytic plaque assay was used to measure cumulative ACTH release from single pituitary cells in culture.

What is the physiological role of intracellular Ca^{2+} as a messenger? What are the feedforward and feedback relationships between major second messengers [cyclic AMP, diacylglycerol (DAG), inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$)] and intracellular Ca^{2+} ? That is, how are individual messenger circuits 'wired' within ACTH cells. Intracellular Ca^{2+} may act as a common signal into which interacting second messenger signals [cyclic AMP, $\text{Ins}(1,4,5)\text{P}_3$, DAG] are transduced and integrated to govern ACTH release. A novel circuit of messenger pathways linked by Ca^{2+} is proposed as the intracellular basis for the synergistic interaction of CRH- and AVP-regulated ACTH release.

Introduction

The way we think about the pituitary gland has been dominated by the emergence and general acceptance of neuroendocrinology. We recognize the hypothalamus as the 'final common pathway', the conductor of the endocrine orchestra. The endocrine hypothalamus elaborates hypophysiotropic hormones

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and sends them to the pituitary *via* the hypophyseal portal vasculature. There is a hierarchy of hypothalamus, pituitary gland and target organs, and thus the idea of hypothalamic supremacy has made good sense for a long time. The brain is the higher seat of learning, its secretions warrant specialized vasculature, the endocrine hypothalamus is poised dominant over the pituitary – it is hard to imagine the pituitary being more than subordinate to hypothalamic hegemony. However, the pituitary gland is not a simple bag of cells, capable of nothing but mechanical responses to stimuli (Leong, Frawley & Neill, 1983). Recent findings suggest that the pituitary is a master gland of underestimated but unmistakable complexity.

Most, if not all, physiological episodes of pituitary hormone secretion can be rationalized in terms of the action of two first messengers acting in concert. Such interactions between regulators of pituitary secretions are rarely simple. For example, it is established that ovarian oestradiol greatly facilitates the action of hypothalamic luteinizing hormone-releasing hormone (LHRH) to induce pituitary luteinizing hormone (LH) secretion. This facilitatory interaction provides the stimulus for the pituitary LH surge that triggers ovulation. Similarly, physiological episodes of pituitary growth hormone (GH) secretion are generally accepted as representing an integration of the inhibitory effects of somatostatin and the stimulatory effects of growth hormone-releasing hormone (GHRH). In a companion chapter (see Thorner, Holl & Leong, 1988), we describe interactions between somatostatin and GHRH that are hierarchic: somatostatin dominates over GHRH in terms of the integrated effects upon cytosolic Ca^{2+} levels and GH secretion (Holl, Thorner & Leong, 1988).

The theme of dualism of first messengers, as well as the inherent complexity of these interactions, probably applies to the regulation of all pituitary hormones. This chapter will review the current understanding of stimulus–secretion coupling in pituitary ACTH cells, particularly from the standpoint of the synergistic interaction between corticotropin releasing hormone (CRH) and vasopressin (AVP). Readers are referred to two excellent reviews concerning adrenocorticotrophic hormone (ACTH) biosynthesis (Lundblad & Roberts, 1988) and the central nervous system regulation of ACTH secretion (Rivier & Plotsky, 1986) that are beyond the scope of this review.

Physiological mechanisms regulating ACTH secretion

One major advance since the primary structure of CRH was sequenced (Vale, Spiess, Rivier & Rivier, 1981) has been the discovery that a subset of hypophysiotropic CRH neurones also contain arginine vasopressin (AVP). AVP and CRH are co-localized in cell bodies originating from the hypothalamic paraventricular nucleus, a finding first evident only in adrenalectomized rats freed from negative glucocorticoid feedback (Roth, Weber & Barchas, 1982; Tramu, Croix & Pillez, 1983; Sawchenko, Swanson & Vale, 1984; Kiss, Mezey & Skirboll, 1984). The studies demonstrated some adaptive plasticity of the contents of CRH neurones,

or at least that the levels of these contents are under regulatory control. Projections of this subset of AVP neurones terminate in the median eminence rather than the posterior pituitary. Recently, in a decisive study of intact rats, applying apparently more sensitive approaches of electron microscopy and immunostaining, it was estimated that about 50 % of hypophysiotropic CRH neurones contained AVP co-localized in the same secretory granules (Whitnall, Mezey & Gainer, 1985; Whitnall, Smyth & Gainer, 1987). Subsets of hypophysiotropic neurones that contain CRH alone, or CRH and AVP in combination, may provide a measure of flexibility in mediating the physiological response to different stressors. Whatever the case, the co-release of hypophysiotropic CRH and AVP is clearly obligatory when neurones co-localizing CRH and AVP are stimulated (Fig. 1). Although this finding places the significance of the synergy between CRH and AVP on a firm hypophysiotropic basis, it is still unclear whether the hypothalamus is the only source of AVP that can influence pituitary ACTH cells.

It has long been recognized that AVP powerfully facilitates CRH-induced ACTH release (Yates *et al.* 1971) by a direct action on the pituitary (Gillies, Linton & Lowry, 1982; Turkelson *et al.* 1982; DeBold *et al.* 1984). In mammals, CRH is the most potent ACTH secretagogue known whereas AVP is a relatively feeble ACTH secretagogue that functions more as a modulator of ACTH release. There is a family of modulators that synergize with CRH, including angiotensin II (Abou-Samra, Catt & Aguilera, 1986; Schoenenberg, Kehrer, Muller & Gaillard, 1987), oxytocin (Antoni, Holmes & Jones, 1983; Gibbs, Vale, Rivier & Yen, 1984; Baertschi & Friedli, 1985), epinephrine and norepinephrine (Vale *et al.* 1983) and C-terminal gastrin-releasing peptide (Hale *et al.* 1984). This family of ACTH modulators that can be matched with CRH may be critical in providing the organism with a flexible response to combat multiple stressful episodes.

Dynamics of CRH-induced ACTH secretion

Pituitary ACTH cells (corticotropes) respond to corticotropin releasing hormone (CRH) with sustained ACTH release in the sustained presence of the stimulus (Fig. 2). Increasing concentrations of CRH elicit a profile of ACTH release that rises in measured increments until a maximal response is achieved. These elegant findings were obtained using a modified microperfusion system that much improved temporal control over the timing of the stimulus presentation and measurement of the hormone response (Watanabe & Orth, 1987). The response measured is the cell population response summed from large numbers of pituitary corticotropes. The question arises: are all the ACTH cells 'turned on' at submaximal doses of CRH? Put another way, how is the graded average population response shown in Fig. 2 built from discrete unitary responses?

A method that measures ACTH release at the level of single cells was developed to answer this question (for a review of the method see Smith, Luque & Neill, 1986; Leong *et al.* 1985). The reverse haemolytic plaque assay (RHPA) quantitatively measures cumulative ACTH release from isolated corticotropes *in vitro*.

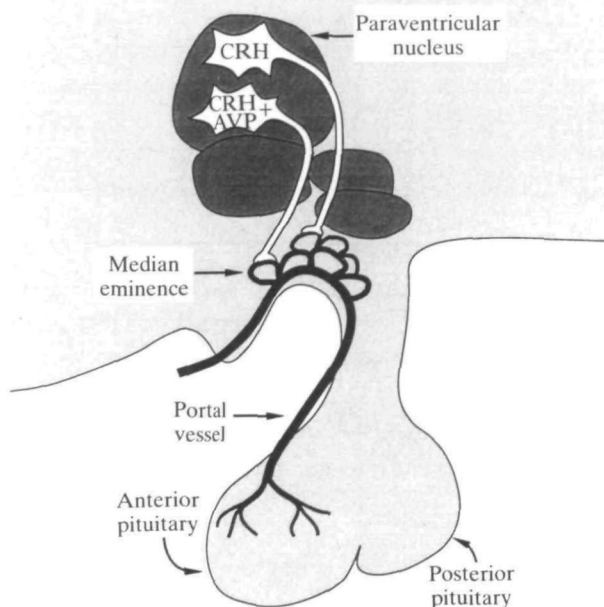


Fig. 1. Schematic representation of CRH-containing neurones in the paraventricular nucleus. Co-localization of CRH and AVP in neuronal subsets.

Batches of pituitary cells were stimulated with different concentrations of CRH for 2 h. The number of cells releasing ACTH was counted and expressed as a percentage of all cells in the mixed-cell pituitary population. Surprisingly, as the CRH concentration increased, so did the total number of cells releasing ACTH. The greatest number of pituitary cells releasing ACTH was 6.0 %, achieved at maximal CRH concentrations (Fig. 3). Longer incubation times did not reveal more ACTH cells. These findings demonstrate that corticotropes respond in a highly individual fashion to CRH. Each corticotrope appears to be endowed with its own activation threshold to CRH. This threshold must be achieved before ACTH release can be initiated. These results suggest that the basis for simple stimulus–secretion coupling is substantially more complex than previously anticipated. Graded tissue responses to increasing concentrations of a stimulus can therefore be explained by different cells in the population breaking through a threshold. That is, a prominent feature of concentration–response curves is the recruitment of ACTH cells into the secretory pool.

The population of ACTH cells thus appears to be highly heterogeneous, comprising individual corticotropes endowed with distinct activation thresholds to CRH. With further analysis, hormone release can also be measured with the RHPA based on the area of the plaque formed around each corticotrope (Fig. 4). Here, there are few surprises since the amount of hormone released from single corticotropes increases as the CRH concentration is raised. The dynamic profile of

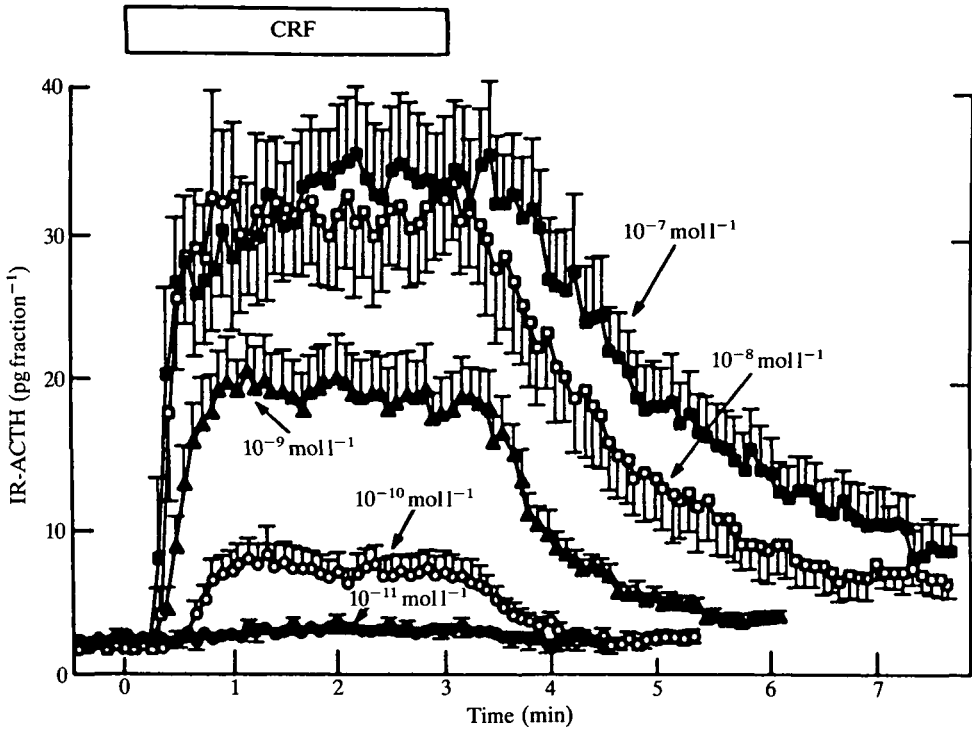


Fig. 2. Detailed profile of ACTH release during sustained CRH treatment. Concentration-response relationships. Note the sustained profile of ACTH release. (Reproduced from Watanabe & Orth, 1987.)

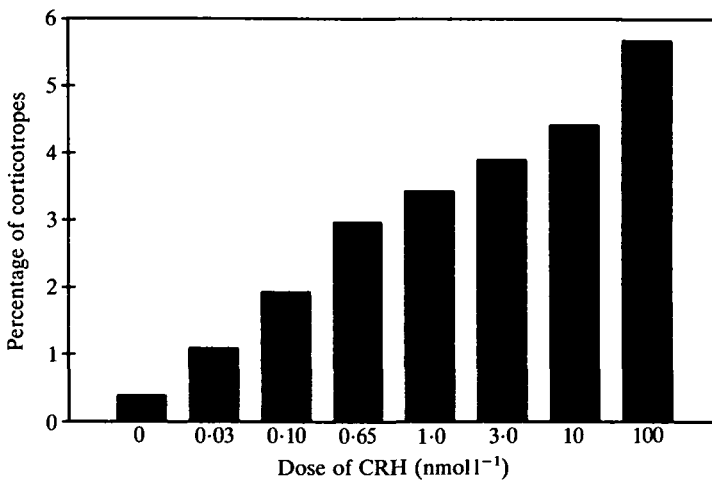


Fig. 3. Fraction of pituitary cells secreting ACTH in response to different concentrations of CRH after 2 h of incubation.

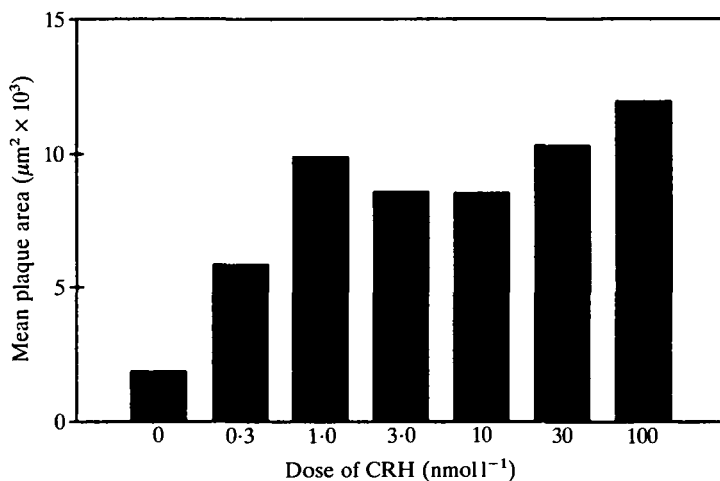


Fig. 4. Mean area (an index of the amount of hormone released) of ACTH plaque formation in response to different concentrations of CRH after 2 h of incubation.

ACTH release from a single cell cannot be measured by RHPA since the technique is limited to the detection of cumulative hormone release occurring over periods of 2–3 h. Although the amount of hormone released from single cells peaks well before saturating CRH concentrations are reached in the example shown (Fig. 4), this is not a consistent finding. Thus, in half the experiments, the mean plaque area continues to increase in measured increments until maximal CRH concentrations are achieved.

Mechanism of CRH-induced ACTH secretion

Role of cyclic AMP

It is well established that CRH increases pituitary cyclic AMP production in both whole and broken cell preparations (Labrie *et al.* 1982; Aguilera *et al.* 1983). Problems of interpretation arising from mixed-cell populations are of less concern here because the actions of CRH on ACTH cells are highly specific. Thus the CRH receptor in the corticotrope is clearly coupled to adenylate cyclase.

Role of cytosolic free Ca²⁺

Cytosolic free calcium levels have been directly measured in single pituitary ACTH cells using the calcium-sensitive fluorescent indicator fura-2 and digital imaging microscopy (Grynkiewicz, Poenie & Tsien, 1985). Routinely, about 15–25 pituitary cells are imaged in a given field using a low-power objective, and the average [Ca²⁺] determined using dual-wavelength ratio imaging. The corticotropes that happen to be in the field are identified retrospectively among the mixed-cell population by RHPA (Leong, 1988). The mean cytosolic free calcium concentration in corticotropes under resting conditions was about 50–100 nmol l⁻¹

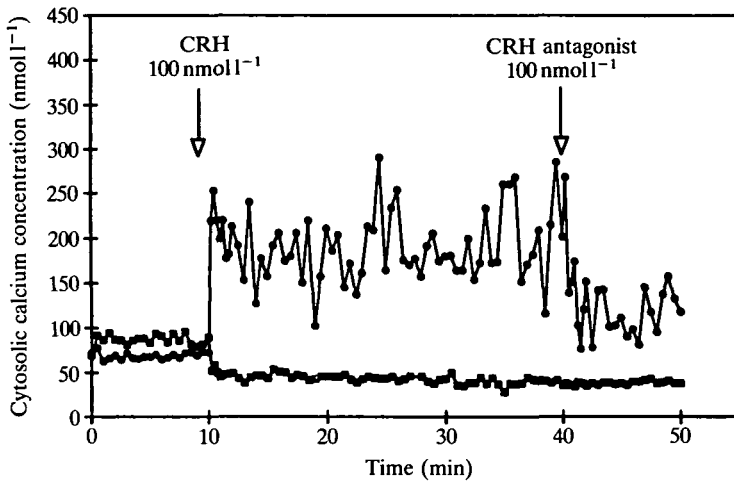


Fig. 5. Representative oscillatory CRH-induced Ca^{2+} responses in a single ACTH plaque-forming cell (●). Depletion of extracellular Ca^{2+} with 5 mmol l^{-1} EGTA (at +10 min) (□) completely abolished CRH-induced Ca^{2+} influx. The antagonist used was alpha-helical CRH.

(Fig. 5). CRH treatment (100 nmol l^{-1}) rapidly stimulated (in 3–50 s) an oscillatory pattern of changing calcium concentration characterized by troughs of 125 nmol l^{-1} and peaks of 300 nmol l^{-1} . This distinctive calcium profile persisted throughout the CRH exposure, and was evident in 28 of 31 corticotropes studied. Treatment with alpha-helical CRH, a potent CRH antagonist, caused calcium levels to subside within 120 s to near basal levels. Removal of extracellular Ca^{2+} completely abolished this profile of CRH-induced Ca^{2+} oscillations. It is noteworthy that CRH-induced Ca^{2+} influx is clearly sustained in the continued presence of the stimulus. This finding parallels the profile of CRH-induced ACTH responses discussed earlier (see Fig. 2).

A strikingly similar profile of oscillating calcium concentration increases was observed in separate experiments, where forskolin was used to bypass the CRH receptor and activate adenylate cyclase directly (Fig. 6). Forskolin-induced calcium concentration rises were also completely abolished by the removal of extracellular calcium. Further, in other studies both CRH- and forskolin-induced $[\text{Ca}^{2+}]$ rises and ACTH secretion were completely abolished by treatment with high concentrations of the calcium channel antagonist verapamil ($100\text{ }\mu\text{mol l}^{-1}$). At high concentrations, verapamil is no longer specific for voltage-sensitive Ca^{2+} channels and acts nonspecifically on other ion channels (see Miller, 1987). Whatever the case, these findings suggest that the CRH-induced cyclic AMP signal must be transduced into a Ca^{2+} signal to initiate ACTH release. Similar conclusions can be made from studies where CRH-induced ACTH release was significantly attenuated with the calmodulin antagonist W7 (which also inhibits protein kinase C) without altering the magnitude of CRH-induced cyclic AMP concentration rises (Murakami, Hashimoto & Ota, 1985a,b).

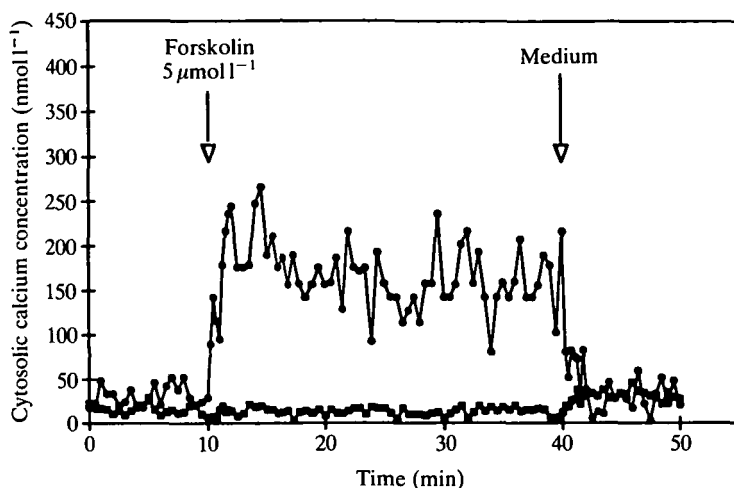


Fig. 6. Representative oscillatory forskolin-induced Ca^{2+} responses in a single ACTH plaque-forming cell (●). Depletion of extracellular Ca^{2+} with 5 mmol l^{-1} EGTA (at -5 min) (□) completely abolished forskolin-induced Ca^{2+} influx (0.4 mmol l^{-1} external Ca^{2+} was reintroduced at $+40 \text{ min}$).

The mechanism of CRH-induced Ca^{2+} influx has been elusive. In patch-clamp studies (Marchetti, Childs & Brown, 1987), L- and T- but not N-type voltage-sensitive Ca^{2+} channels (Nowarck, Fox & Tsien, 1985) were demonstrated in pituitary corticotropes previously identified using a biotinylated labelled CRH fluorescent probe (Childs, Unabia, Burke & Marchetti, 1987b). Experiments with dihydropyridines have produced mixed results. Thus, nimodipine, the 'pure' enantiomer (–)R202-791, and nifedipine (albeit in large amounts) effectively abolished CRH-induced ACTH release (Childs, Marchetti & Brown, 1987a; Murakami, Hashimoto & Ota, 1985a,b). However, other conflicting reports indicate that nitrendipine merely altered the sensitivity of the response (Abou-Samra *et al.* 1987a), or that verapamil had no effect on ACTH release (Giguere, Lefevre & Labire, 1982). One potential problem is that cyclic AMP levels have not always been measured. For example, large concentrations of nifedipine not only inhibited CRH-induced ACTH release but also inhibited cyclic AMP production. Thus, in some studies the effect of nifedipine in blocking ACTH secretion can be attributed to the inhibition of cyclic AMP production and not necessarily to an action on voltage-sensitive Ca^{2+} channels (Murakami, Hashimoto & Ota, 1985a,b).

It has been reported (Childs *et al.* 1987a) that tetrodotoxin completely abolished CRH-induced ACTH release, suggesting the involvement of voltage-sensitive sodium channels. These authors suggested that CRH might elicit a change in membrane potential in corticotropes obtained from normal tissue. The following two possibilities were discussed: (1) hyperpolarization might remove tonic inactivation of Na^{+} channels and increase the number of channels available and (2) depolarization might activate enough Na^{+} channels to produce an action

potential, thus depolarizing the cell to a value that would open L-type Ca^{2+} channels. Current schemes are necessarily broad because the precise electrophysiological correlates of CRH action in any pituitary corticotrope are presently unknown.

This discussion has deliberately focused on studies with cells derived from normal tissue. Problems arising from the use of mixed-cell pituitary populations are only gradually being overcome by single-cell studies (RHPA, patch-clamp techniques, fura-2 fluorescence measurements). Substantial biochemical and electrophysiological studies have been performed on the pituitary AtT-20 mouse pituitary tumour cell line that is believed to be a relatively homogeneous population of cells. However, AtT-20 cells possess CRH but not AVP receptors and, unlike corticotropes obtained from normal tissue, anomalously express inhibitory somatostatin receptors (Axelrod & Reisine, 1984). Using intracellular microelectrodes, most AtT-20 cells have been shown to display spontaneous action potential activity that persisted in tetrodotoxin- and Na^{+} -free solutions but which was depressed or abolished in cobalt- or Ca^{2+} -free solutions (Suprenant, 1982). These findings suggest a role for Ca^{2+} as the major inward-carrying current source during action potential generation. Although the effects of CRH are presently unknown, the stimulatory effect of isoproterenol increased the frequency of action potentials without changing the resting potential or input resistance. In AtT-20 cells isoproterenol and CRH both increase cyclic AMP production. Thus the electrophysiological effects of β -adrenergic stimulation may resemble the action of CRH. These findings were essentially confirmed and extended in patch-clamp studies of AtT-20 cells (Adler *et al.* 1983). In this report, action potentials were shown to have discrete Na^{+} and Ca^{2+} components and the bursts were separated by a Ca^{2+} -sensitive after-hyperpolarization. Single-channel recordings revealed that the after-hyperpolarization is mediated by Ca^{2+} -dependent K^{+} channels.

Calcium measurements in bulk AtT-20 cell suspensions have demonstrated that CRH promotes Ca^{2+} influx, an effect mimicked exactly by forskolin and the cyclic AMP analogue 8-bromo-cAMP (Luini *et al.* 1985). CRH- and 8-bromo-cAMP-induced Ca^{2+} influx was attenuated with the dihydropyridine nifedipine, nisoldipine, and also with verapamil. In addition, these authors exploited whole-cell patch-clamp techniques to show that 8-bromo-cAMP increased voltage-dependent Ca^{2+} currents. Furthermore, phorbol ester (PMA) treatment decreased cytosolic Ca^{2+} levels (Luini *et al.* 1985; Lewis & Weight, 1988). In further studies, an essential role of cyclic AMP and cyclic-AMP-dependent phosphorylation was suggested by the finding that incorporation of a cyclic-AMP-dependent A-kinase inhibitor (Walsh–Apelman–Krebs inhibitor) into the cell *via* liposomes significantly attenuated CRH-induced ACTH release (Reisine, Rougon, Barbet & Affolter, 1985). Similar studies measuring cytosolic Ca^{2+} levels in bulk AtT-20 cell suspensions demonstrated that CRH- or forskolin-induced rises in cytosolic $[\text{Ca}^{2+}]$ were completely abolished by inhibitors of cyclic-AMP dependent A-kinase action (Guild & Reisine, 1987). These findings provide strong evidence for the existence of second messenger (e.g. cyclic AMP) operated ion channels. The site of A-

kinase phosphorylation is presently unknown. Interestingly, some voltage-activated Ca^{2+} channels in pituitary cells must be phosphorylated to become responsive to membrane depolarization (Armstrong & Eckert, 1987). However, A-kinase may not necessarily phosphorylate Ca^{2+} channels directly, as has been proposed for cardiac cells (Osterrieder *et al.* 1982). Thus, cyclic AMP might, for example, reduce the effectiveness of a K^{+} channel, prolonging the depolarizing phase of each action potential (e.g. *Aplysia*, Shuster, Camardo, Siegalbaum & Kandel, 1985; Avenet, Hoffman & Lindemann, 1988), activate chloride channels (Li *et al.* 1988) or activate Na^{+} channels (e.g. olfactory receptor cells, Gold & Nakamura, 1987). The prospects are complex because, in addition to voltage-gated channels (sensitive to changes in membrane potential) and ligand-gated channels (directly gated or second-messenger-gated), it is now recognized that G-protein-gated ion channels also exist (Birnbaumer & Brown, 1988). It is quite possible that two or three such mechanisms co-exist to create multiple signalling pathways leading to CRH-induced Ca^{2+} influx. Bifurcating signal-transduction mechanisms would be expected to provide the flexibility necessary for complex control mechanisms governing stimulus-secretion coupling in pituitary corticotropes. Further studies are required to unravel the complex mechanism underlying CRH-induced Ca^{2+} influx.

Dynamics of AVP-induced ACTH secretion

Microperfusion studies have demonstrated that AVP stimulates a transient profile of ACTH release in the sustained presence of the stimulus (Fig. 7). This profile contrasts with the sustained ACTH response elicited during the sustained presence of a CRH stimulus.

Mechanisms of AVP-induced ACTH secretion

Role of cyclic AMP

In dissociated pituitary cells, AVP has no effect on pituitary cyclic AMP levels under conditions where significant ACTH release can be demonstrated. Interestingly, different effects sometimes occur if pituitary quarters are used. Thus AVP is reported to increase cyclic AMP concentration (Rose & Conklin, 1978; Knepel, Homolka, Vlaskovska & Nutto, 1984) although this is controversial (Murakami *et al.* 1985*b*). Although these differences are difficult to resolve completely, it is intriguing to consider that cell-cell interactions or paracrine effects may be critical for AVP to elicit pituitary cyclic AMP concentration increases.

Role of phosphoinositide metabolism

There is evidence that AVP action activates phosphoinositide metabolism. AVP increases turnover of polyphosphoinositides (Raymond, Leung, Veilleux & Labrie, 1985) and, more directly, AVP-induced increases of inositol polyphosphate production in mixed pituitary cell populations (Todd & Lightman, 1987).

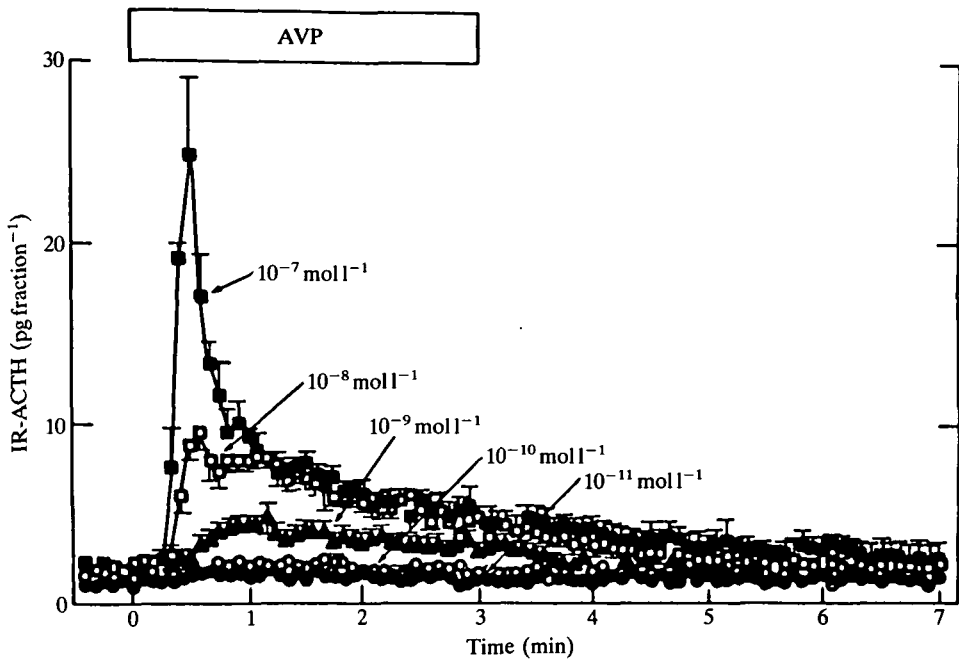


Fig. 7. Detailed profile of ACTH release during sustained AVP treatment. Concentration–response relationships. Note the transient profile of ACTH release. (Reproduced from Watanabe & Orth, 1987.)

AVP-induced inositol polyphosphate production was completely abolished by a V1 receptor antagonist and by adrenalectomy. The major *caveat* is that we cannot be certain that the effects of AVP, as measured in mixed pituitary cell populations, are specific for the pituitary corticotrope.

Role of Ca^{2+}

It can be clearly demonstrated that sensitivity to both CRH and AVP can occur within the same corticotrope population. AVP rapidly mobilizes a transient release of Ca^{2+} from an intracellular pool in single ACTH plaque-forming cells previously stimulated with CRH (Fig. 8). In the continued presence of AVP, a second plateau phase of lower magnitude can be attributed to the influx of extracellular Ca^{2+} . In CRH-sensitive corticotropes, this spike and plateau response pattern did not appear to be altered by the V1 antagonist [$d(\text{CH}_2)_5, \text{Tyr}(\text{Me})$]-AVP. High concentrations of verapamil ($100 \mu\text{mol l}^{-1}$) or low extracellular Ca^{2+} levels had no effect on the first phase but completely abolished Ca^{2+} influx. It is established that the common mechanism of releasing intracellular Ca^{2+} stores is triggered by inositol 1,4,5-trisphosphate in a host of different cell types (Berridge & Irvine, 1984; Berridge, 1987). These findings provide strong evidence indicating that the corticotrope AVP receptor is indeed linked to the activation of phospholipase C. If the corticotrope AVP receptor were linked

to adenylate cyclase activation, the transient profile of changing $[Ca^{2+}]$, as previously observed with CRH or forskolin, would have been anticipated (see Figs 5, 6). Interestingly, the AVP-induced Ca^{2+} response profile determined in single cells appears to match the ACTH release profile measured previously in population studies (Fig. 7). Thus, there are striking similarities between the Ca^{2+} response and the corresponding profile of ACTH release.

Mechanism of facilitation: the interaction between CRH and AVP

It has been reported that AVP potentiates CRH-induced cyclic AMP production (Giguere & Labrie, 1982; Labrie, Giguere, Proulx & Lefevre, 1984), although not in broken cell preparations (Gaillard *et al.* 1984). Phorbol esters also potentiate CRH-induced cyclic AMP production (Cronin, Zysk & Baertschi, 1986; Abou-Samra *et al.* 1987b). Phorbol esters, to some extent, mimic the action of diacylglycerol in activating protein kinase C (Nishizuka, 1984). This synergistic

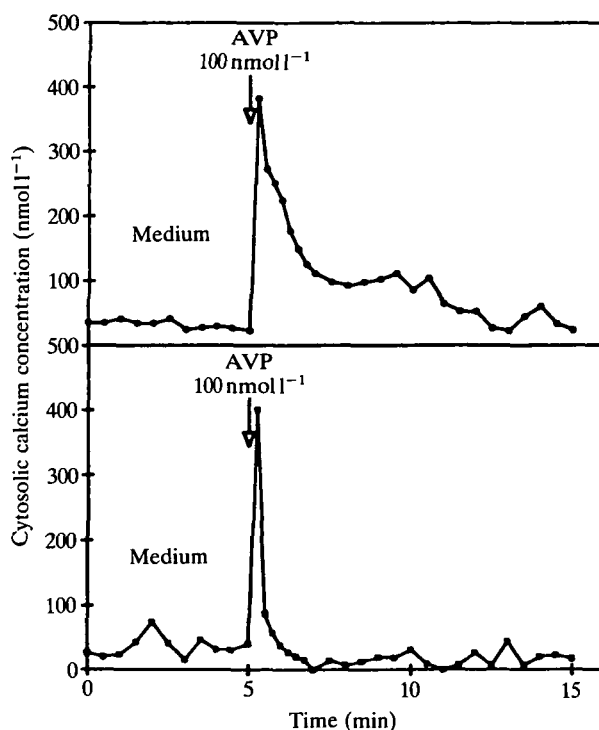


Fig. 8. Representative AVP-induced Ca^{2+} responses in a single ACTH cell previously induced to form a plaque with CRH. The rapid first phase appears to represent Ca^{2+} mobilization from internal stores followed by a plateau phase of Ca^{2+} influx (top panel). Depletion of extracellular Ca^{2+} with 5 mmol l^{-1} EGTA had no effect on the magnitude of AVP-induced Ca^{2+} mobilization but completely abolished the plateau phase of Ca^{2+} influx (lower panel).

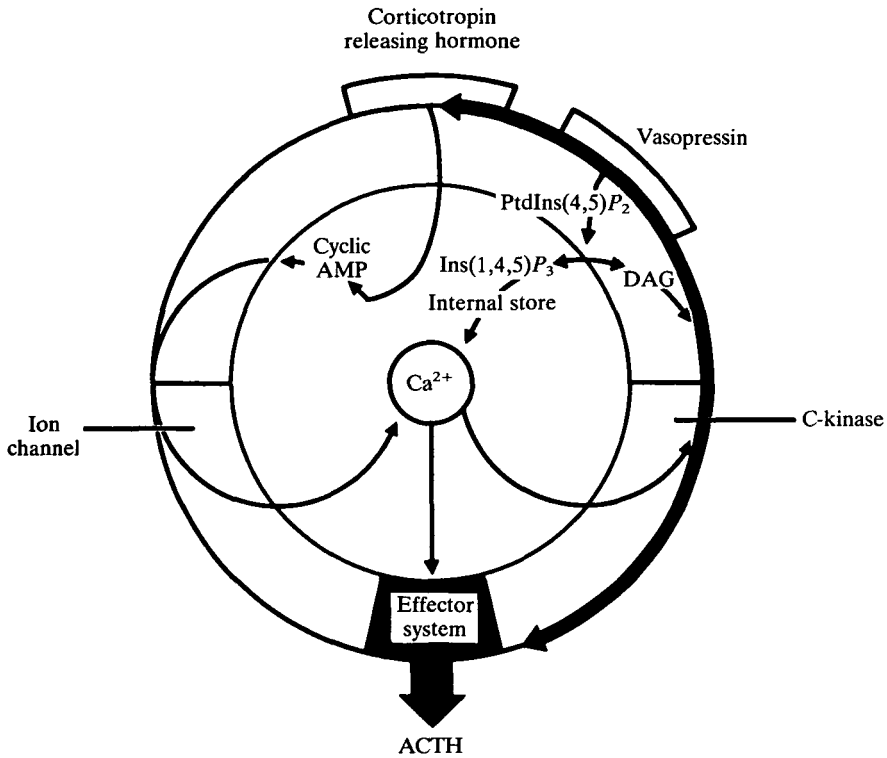


Fig. 9. The second messenger 'loop' hypothesis for the synergistic interaction between AVP and CRH in anterior pituitary corticotropes. There is a circuit of second messengers [Ins(1,4,5)P₃, DAG, cyclic AMP] linked by cytosolic Ca²⁺ to form a positive feedback loop.

interaction between protein kinase C activation and CRH-induced cyclic AMP production in pituitary corticotropes has also been reported in a number of other cell types, but is not universal (see Langlois, Saez & Begeot, 1987). Cross-talk between these two messenger systems is an important part of the mechanism whereby AVP facilitates CRH-induced ACTH release.

The following working hypothesis is currently under investigation in this laboratory. The model essentially comprises a circuit of second messenger pathways linked by cytosolic Ca²⁺ to form a positive feedback loop (Fig. 9). (1) CRH activates adenylate cyclase to trigger cyclic AMP production. (2) Cyclic AMP is transduced into Ca²⁺ influx. (3) AVP activates phospholipase C to produce inositol trisphosphate (InsP₃) and diacylglycerol (DAG). (4) InsP₃ elicits a large transient discharge of Ca²⁺ from internal stores that may facilitate the recruitment of cytosolic C-kinase (and/or phospholipase C) to the membrane. (5) Diacylglycerol activates protein kinase C in the plane of the membrane. (6) C-kinase activation is potentiated by ever-increasing levels of cytosolic Ca²⁺. (7) C-kinase potentiates CRH-induced cyclic AMP production. (8) C-kinase

activation increases the responsiveness of the effector system that translates the Ca^{2+} signal into exocytosis and ACTH release.

Synergy between interacting pairs of messengers may take place at two intracellular steps: Ca^{2+} and DAG interact to activate C-kinase and, in turn, C-kinase potentiates CRH-induced cyclic AMP production. Thus, even though the 'strength' of the external stimulus (CRH and AVP) remains constant, the synergy generated from interacting intracellular second messengers progressively leads to ever-increasing cytosolic Ca^{2+} levels; this in turn facilitates further C-kinase activation, in turn more cyclic AMP production, causing more Ca^{2+} influx completing the circle. Element 4 above may be critical since CRH/AVP-induced cyclic AMP production is not synergistic in broken cell preparations: that is, conditions where the recruitment of cytosolic C-kinase to the membrane (or possibly phospholipase C) cannot occur. To some extent positive feedback loops represent a homeostatic hazard. Therefore a means to short-circuit this second messenger 'loop' may exist to extinguish the reaction. Alternatively, the system may simply continue to run until a key metabolite becomes exhausted.

Final remarks

These studies have characterized the Ca^{2+} responses associated with AVP (phosphoinositide metabolism) or CRH (adenylate cyclase) stimulation in single CRH-sensitive ACTH cells. Both pathways of receptor-mediated signal transduction include Ca^{2+} signalling in their mechanism of action in the corticotrope. The striking overlap of Ca^{2+} signalling profiles with the dynamic pattern of ACTH release is impressive, suggesting an intimate relationship between Ca^{2+} signalling and the release process.

The proposed second messenger 'loop' hypothesis that attempts to account for the facilitatory effect of AVP upon CRH-induced ACTH secretion may not be exclusive (Fig. 9). For example, it has been noted recently that the synergy of CRH/AVP interactions is most prominent in *in vivo* studies or studies where pituitary fragments have been used (Schwartz & Vale, 1988). Thus it is not clear if all the mechanisms governing AVP/CRH interactions are expressed in isolated pituitary cells. Moreover, there is intriguing new evidence supporting the concept that AVP-induced ACTH release may occur in a subpopulation of corticotropes that appears to be unresponsive to CRH (Schwartz & Vale, 1988). These findings, if confirmed, indicate yet another level of complexity in the regulation of anterior pituitary corticotrope function. This issue is not addressed in the studies described here of CRH-responsive corticotropes. The potential functional heterogeneity of the corticotrope subpopulation is currently under investigation. Clearly, single-cell studies promise to provide new insights into the regulation of anterior pituitary hormone function.

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