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ORIGINAL ARTICLE

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# Direct actions of adrenergic agents on rat anterior pituitary cells

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**Abstract** We studied the effects of adrenergic agents on the five main cell types of the rat anterior pituitary by monitoring the changes of the cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in single cells that were identified by multiple sequential primary immunocytochemistry at the end of the $Ca^{2+}$ measurements. Adrenaline (100 nM) increased $[Ca^{2+}]_i$ in 30% of the cells. Responses were most prominent in somatotrophs and corticotrophs (40–65% of the cells responded) whereas the other three cell types, lactotrophs, thyrotrophs and gonadotrophs, gave poorer responses. Selective agonists and antagonists revealed the presence of both $\alpha_1$- and β-adrenergic receptors. $\alpha_1$-Receptors dominated in corticotrophs, β-receptors in somatotrophs. The $\alpha_1$-adrenergic responses increased with culture of the cells. The β-adrenergic responses were mediated by cAMP and consisted of stimulation of $Ca^{2+}$ entry through L-type voltage-gated channels. Stimulation of $\alpha_1$-receptors released $Ca^{2+}$ from intracellular stores in corticotrophs and induced cAMP-independent $Ca^{2+}$ entry in somatotrophs. The effects of $\alpha_1$-agonists were additive with those of the releasing factors growth hormone-releasing hormone (GHRH) and corticotropin releasing factor (CRF) whereas those of the β-agonists were not. Our results suggest that direct effects of plasma catecholamines on AP cells may contribute to the hormonal response to stress.

**Keywords** Pituitary cells · Somatotrophs · Corticotrophs · Catecholamines · Adrenaline · Calcium influx · Growth hormone · ACTH

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## Introduction

In 1947, Long proposed that circulating adrenaline could stimulate adrenocorticotropic hormone (corticotropin, ACTH) secretion, thus reinforcing the response to stress [23]. It was reported later that adrenaline induces secretion of ACTH [15, 27, 39, 45] and that stress increases the catecholamine content of hypophysial portal blood [18, 24, 34, 44]. However, later results of in vivo experiments in different species and using different adrenergic agonists are conflicting (see [2]). The interpretation of such results is complicated because of possible actions at two different targets, the hypothalamus and the own anterior pituitary (AP) cells, and mediation by different adrenergic receptor types.

In the corticotroph cell line AtT-20, β-adrenergic stimulation induces expression of the *pro-opiomelanocortin* (*POMC*) gene and secretion of ACTH by a cAMP-mediated mechanism [4, 5, 37]. Cell electrical activity and $Ca^{2+}$ entry are also stimulated by β-adrenergic agonists [25]. The presence of β-adrenergic receptors has been documented by immunocytochemistry of the AP [31] and in rat corticotrophs [40]. Adrenaline and isoproterenol induce ACTH release in vivo and this effect is blocked by the β-adrenergic antagonist propranolol [45]. Adrenaline also induces release of ACTH from cultured AP cells [47], but this effect is not blocked by propranolol [16]. In another study the ACTH response to β-adrenergic agonists was qualified as equivocal [32].

$\alpha_1$-Adrenergic receptor agonists induce oscillations of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and ACTH secretion in rat AP cells in primary culture [17, 46] and in man in vivo [2]. However, $\alpha_1$-receptors have not been detected by autoradiographic procedures in the intact AP [8, 13], suggesting that the effect observed in vivo could be mediated by the hypothalamus and that primary culture might favour expression of $\alpha_1$-receptors [2].

Both $\alpha_1$- [26, 36, 41] and β- [20, 32, 41] adrenergic agonists reportedly stimulate growth hormone (GH) secretion from AP cells in primary culture. $\alpha_1$-Agonists increase cell $Ca^{2+}$ [30]; β-agonists increase cAMP produc-

tion [10]. In humans, central $\alpha_2$-adrenoceptors increase growth hormone-releasing hormone (GHRH) secretion whereas β-adrenoceptors increase somatostatin secretion, and this may by the cause of variable results in vivo [2]. In dissociated goldfish pituitary cells the $\alpha_2$-receptor agonists decrease both basal GH release [22] and secretion evoked by either adenylate cyclase activators, protein kinase activators or $Ca^{2+}$ ionophores [52]. Inhibitory effects of $\alpha_2$-agonists have also been reported in ovine pituitary cells [41] and in vivo, although it is not clear in the latter case whether the effects were direct or mediated through the hypothalamus [2].

The results reported for regulation of the secretion of other AP hormones are even more scarce and contradictory. $\alpha_1$-Agonists induce secretion of thyrotrophin (TSH) and prolactin (PRL) in man, but this effect seems to be mediated through the hypothalamus [2]. In cultured rat cells, however, $\alpha_1$-agonists modulate TSH secretion [33, 43]. β-Agonists reportedly induce PRL secretion in vivo and in pituitary cell aggregates [6, 7, 14, 37], although this effect could not be confirmed in dissociated AP cells [32]. Modulation of luteinising hormone (LH) secretion by catecholamines has been reported in the rabbit in vivo, although no clear effects could be documented in humans [2], perfused rat pituitary [19] or dissociated AP cells [32].

Immunocytochemistry and autoradiography have demonstrated the presence of β- but not α-adrenergic receptors in the AP [2, 8, 12, 13]. However, both α- and β-receptors can be detected in AP cells in primary culture [12, 31, 40], suggesting that adrenergic receptor expression may change during culture.

$Ca^{2+}$ is a universal messenger for cell activation [9]. The increase of $[Ca^{2+}]_i$ is the main trigger for regulated exocytosis in many tissues, including the pituitary. An increase in $[Ca^{2+}]_i$ may be triggered by activation of plasma membrane $Ca^{2+}$ channels or by release of $Ca^{2+}$ from intracellular stores [3, 9] and many of the regulators of AP hormone secretion, including the hypothalamic releasing hormones, are known to act through one or both of these pathways [11, 21, 28, 35, 38, 42]. In addition, $Ca^{2+}$ also regulates long-term effects acting at the gene expression level [9].

In the present study the effects of adrenaline and selective $\alpha_1$- and β-adrenergic receptor agonists were studied systematically in freshly isolated rat AP cells and after 24 h in primary culture. Changes of $[Ca^{2+}]_i$ were followed at the single-cell level and used as an index of cell activation. When $[Ca^{2+}]_i$ changes were detected, the origin of $Ca^{2+}$, either entry from the extracellular medium or release from the intracellular stores, was investigated. Multiple sequential primary immunocytochemistry (MSPI) was performed following the $[Ca^{2+}]_i$ measurements, this allowing classification of the cell responses for the different AP cell types.

## Materials and methods

AP cells were prepared from 8- to 10-week-old male Wistar rats as described previously [48, 51] and grown in RPMI 1640 medium containing 10% fetal calf serum over glass cover-slips coated with poly-L-lysine (0.01 mg/ml, 10 min). After either 1 h (0 days in vitro, 0 DIV) or 24 h in culture (1 DIV), cell-coated cover-slips were loaded with fura-2 by incubation with 4 μM fura-2/AM at room temperature for 1 h. Measurements of $[Ca^{2+}]_i$ and time-resolved digital image analysis were performed as described before [1, 48, 51]. Experiments were performed at 37 °C. The standard incubation solution had the following composition (in mM): NaCl, 145; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; sodium-HEPES, 10, pH 7.4. Cells were perfused continuously at 2–3 ml/min and test substances were added to the perfusion solutions.

At the end of the $[Ca^{2+}]_i$ measurements, AP cells in the microscope field were typed by MSPI using antibodies raised against pituitary hormones [1, 49, 50, 51]. The use of primary antibodies allows fast processing, necessary for in situ analysis, and circumvents the cross-reactions that are unavoidable when using secondary antibodies with multiple primaries. In addition, as there is no need to move the preparation during the procedure, image-processing techniques can be used with MPSI to improve analysis. Fluorescent antibodies were prepared from antisera provided by the National Institute of Diabetes and Digestive and Kidney Diseases and purified over a protein A-sepharose column [49, 51] and the reaction times shortened as much as possible. Briefly, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min, permeabilized with 0.3% Triton X-100 in PBS for 3 min and washed with PBS for 5 min. Then, 10% goat serum in PBS was added. After 5 min the antibody against one of the AP hormones, labelled with Oregon green 488, was added and the incubation continued for 15–30 min. After washing, a fluorescence image was captured (excitation, 490 nm; emission, >510 nm) with the image processor. The process was repeated with a second and a third fluorescent antibody. Specific staining with each antibody was obtained by subtracting from each image the previous one. Finally, nuclei were stained with Hoechst 33258 (0.5 μg/ml, 10 min) and another fluorescence image was acquired (excitation, 340 nm; emission, 450 nm). The image from the fluorescently stained nuclei facilitated definition of cellular boundaries in cells that were physically close. In routine experiments, antibodies against ACTH, GH and PRL were used in this sequence. This allowed definition of four cell types: ACTH+, GH+, PRL+ and the remainder, which must include thyrotrophs and gonadotrophs.

For cAMP measurements, AP cells, plated at $5 \cdot 10^5$ cells/well, were first incubated with standard incubation medium for 30 min at 37 °C and then for 15 min with the adrenergic agonists. The incubation period was terminated by addition of ethanol-EDTA. cAMP was determined using the cyclic AMP [$^3$H] assay system kit (Amersham Ibérica, Madrid, Spain).

Antisera against rat prolactin (rabbit, AFP425-10-91) and GH (monkey, AFP4115) and anti-human ACTH (rabbit, AFP39013082) were generous gifts from the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), the National Institute of Child Health and Human Development and the US Department of Agriculture, (Rockville, Md., USA). Furnidipine was a generous gift from Laboratorios Alter (Madrid, Spain). Fura-2/AM and Oregon green 488-isothiocyanate were purchased from Molecular Probes (Eugene, Ore., USA). Other chemicals were obtained either from Sigma (Madrid, Spain) or from E. Merck (Darmstadt, Germany).

## Results

Stimulation of AP cells with adrenaline (100 nM) increased $[Ca^{2+}]_i$ in some of the cells. Fig. 1 shows the averaged trace of all the cells responding to the agonist present in the same microscope field (Fig. 1A) and the traces corresponding to two individual cells (Fig. 1B and C). The increase of $[Ca^{2+}]_i$ began a few seconds after

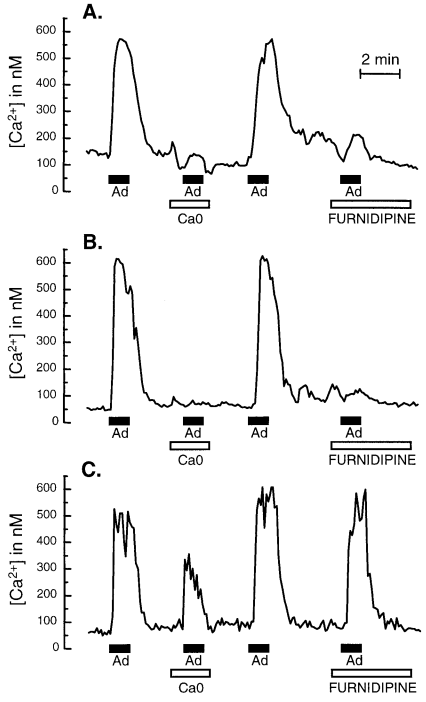


**Fig. 1A–C** Adrenaline (*Ad*, 0.1 µM) increases cytosolic free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in rat anterior pituitary (AP) cells. **A** Average of 28 adrenaline-sensitive cells present in the same microscope field of view. The $[Ca^{2+}]_i$ measured for each cell were added and divided by the number of cells. **B**, **C** Two representative single cells. $Ca^{2+}$-free medium (*Ca0*) contained 0.5 mM EGTA. The concentration of furnidipine was 1 µM

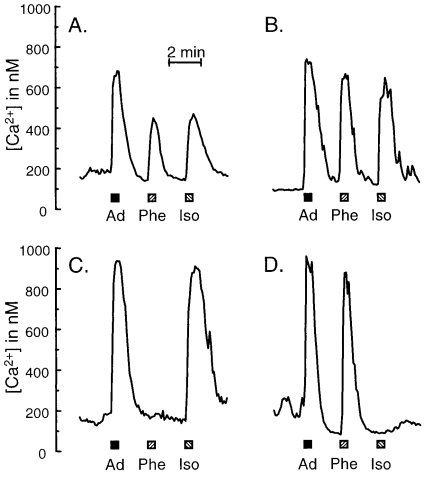


**Fig. 2A–D** AP cells possess $\alpha_1$- and β-adrenergic receptors. The concentrations of adrenaline, phenylephrine (*Phe*) and isoproterenol (*Iso*) were 0.1, 1 and 1 µM, respectively. **A** Averaged response of 15 single cells. **B–D** Three representative single cells. Representative of 19 similar experiments

stimulation and reached its maximum within about 20 s. On washing the adrenaline out, $[Ca^{2+}]_i$ returned to the resting level and a second stimulation with the drug produced a similar effect. In a pool of 1640 cells studied in 20 different experiments, 590 (36%) cells responded to adrenaline (a response being defined as $\Delta[Ca^{2+}]_i$>50 nM after stimulation with the agonist). Thus, our results indicate that one out of every three cells in the AP possessed functional adrenergic receptors. Since the AP cells used in our routine experiments had been maintained in primary culture for 1 day (1 DIV), it could be argued that culture conditions had promoted expression of adrenergic receptors not present in the intact gland. To address this question, we repeated the experiments using freshly isolated cells (0 DIV, see Materials and methods). In a pool of 1376 cells analysed in ten different experiments 385 (28%) responded to adrenaline with an increase of $[Ca^{2+}]_i$. These results indicate that the ability to respond to adrenaline is a genuine property of a significant fraction of AP cells and not an artefact induced by cell culture.

To investigate the mechanisms of the adrenaline-induced $[Ca^{2+}]_i$ increase we compared the effects obtained in regular $Ca^{2+}$-containing medium and in $Ca^{2+}$-free medium. The average $[Ca^{2+}]_i$ response was very much reduced by removal of extracellular $Ca^{2+}$ (Ca0 in Fig. 1A) and reappeared on $Ca^{2+}$ addition. Furnidipine (1 µM), a dihydropyridine antagonist of L-type voltage-gated $Ca^{2+}$ channels [24], had much the same effect as $Ca^{2+}$ removal. Single-cell analysis showed that $Ca^{2+}$ removal abolished the response in 86% of the adrenaline-sensitive cells (*n*=24) and furnidipine did so in 76% (*n*=21). Figure 1 B and C illustrates both responses at the single-cell level. Our results thus indicate that $Ca^{2+}$ entry through voltage-gated channels accounts for the increase of $[Ca^{2+}]_i$ induced by adrenaline in most of the responding cells. Adrenaline, however, is able to induce $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores in a minor fraction of cells.

To establish the type of adrenergic receptors involved in the actions of adrenaline, the effects of the receptor-selective agonists phenylephrine ($\alpha_1$) and isoproterenol (β) were studied. Figure 2 compares the effects of adrenaline (0.1 µM, Ad), phenylephrine (1 µM, Phe) and isoproterenol (1 µM, Iso). Figure 2A shows the averaged trace of 15 adrenaline-sensitive cells present in the same microscope field. Both $\alpha_1$- and β-agonists increased $[Ca^{2+}]_i$. At the single-cell level all possible combinations of receptors were found; some cells responded to both agonists (Fig. 2B), while others responded only to isoproterenol (Fig. 2C) or to phenylephrine (Fig. 2D). In 4570 cells analysed in 40 different experiments 34% of cells responded to phenylephrine, 24% to isoproterenol and 12% (included in the previous two categories) responded to both. The use of selective antagonists confirmed the presence of $\alpha_1$- and β-receptors (Fig. 3). The β-blocker propranolol selectively abolished the response to isoproterenol (Fig. 3A) whereas the $\alpha_1$-blocker prazosin selectively prevented the response to phenylephrine (Fig. 3B).

The $\alpha_2$-adrenoceptor agonist clonidine produced by itself a small increase of $[Ca^{2+}]_i$ in a minor fraction of the cells. However, when given before isoproterenol or phenylephrine it antagonized the increase of $[Ca^{2+}]_i$ in-

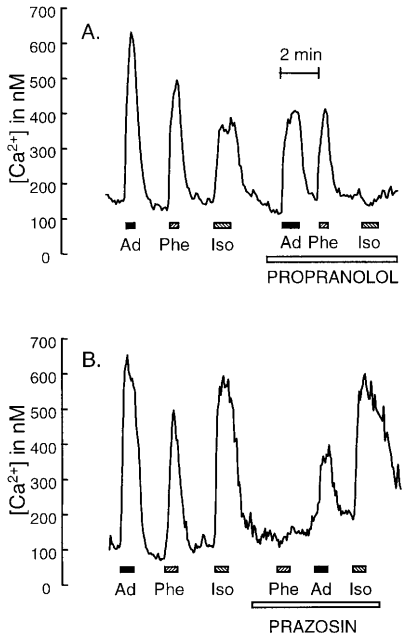


**Fig. 3A, B** Inhibition of the effects of adrenergic agonists by propranolol (5 μM) and prazosin (0.1 μM). Details as in Fig. 2. The traces shown are the average of 30 (**A**) and 18 (**B**) adrenaline-sensitive cells. Representative of three similar experiments

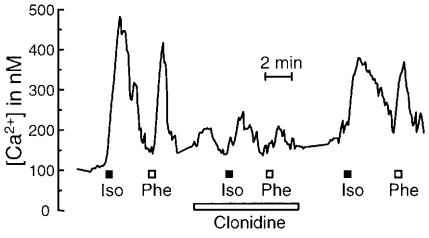


**Fig. 4** Clonidine (1 μM) antagonizes the effects of isoproterenol and phenylephrine. Details as in Fig. 2. The traces shown are the average of 16 cells that responded to both agonists. Representative of two similar experiments

duced by these agonists and this effect was reversed by washing the agent out (Fig. 4). In 32 isoproterenol-sensitive cells clonidine decreased the response, measured as the mean (±SEM) peak $\Delta[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_{i,max}$), from 407±31 to 160±32 nM. In 41 phenylephrine-sensitive cells clonidine decreased the $[Ca^{2+}]_i$ response from 400±28 to 143±23 nM.

Our primary cultures contain at least five different AP cell types, each storing a different AP hormone: somatotrophs, storing GH; lactotrophs, storing PRL; corticotrophs, storing ACTH; gonadotrophs, storing gonadotrophins (follicle-stimulating hormone, FSH, and LH) and thyrotrophs, storing TSH. The percentages of each cell type are 40, 58, 18, 8, and 2%, respectively, whereby some cells show reactivity for more than one hormone [50, 51]. To establish whether or not the adrenergic receptors were distributed homogeneously among the different AP cell types, we followed the $[Ca^{2+}]_i$ responses

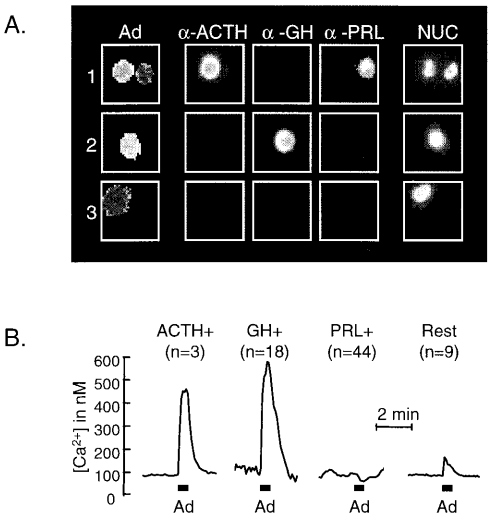


**Fig. 5A, B** Response to adrenaline in the different AP cell types. **A** Identification procedure employing multiple sequential primary immunocytochemistry (MSPI; see Materials and methods). The *first column* shows a fura-2 ratio image after stimulation with adrenaline (0.1 μM), coded in a grey scale: the brighter the pixel the higher the $[Ca^{2+}]_i$. *Columns 2–4* show specific staining with three different antibodies against AP hormones (*ACTH* adrencorticotropic hormone, *GH* growth hormone, *PRL* prolactin). The *last column* shows nuclear (*NUC*) staining. Each row shows the same area (30×30 μM) of the microscopic field of view. **B** Responses to adrenaline averaged for all the cells of each type present in the same microscope field; *n* number of cells. The group labelled *Rest* comprises those cells not stained by the three specific antibodies

to adrenaline in all cells present in the microscope field and then employed MSPI to identify each cell. In routine experiments three antibodies were employed, anti-PRL, anti-GH and anti-ACTH. This allowed classification of the cells into four groups: PRL+ (lactotrophs), GH+ (somatotrophs), ACTH+ (corticotrophs) and the remainder (which must include the thyrotrophs and gonadotrophs). Figure 5A illustrates the basis of this procedure. Each line corresponds to the same area (30×30 μM) of the microscope field. The first column (Ad) shows a pseudocolour image of the fura-2 fluorescence ratio taken at the time of stimulation with adrenaline. The three subsequent columns (α-ACTH, α-GH and α-PRL) show specific fluorescence with each one of the three antibodies used. The last column (NUC) shows nuclei, stained to enable the cells present in the field to be located. In line 1 the cell on the left was a corticotroph (α-ACTH) and responded to adrenaline (Ad) whereas the one in the right was a lactotroph (α-PRL) and did not respond to adrenaline. Line 2 shows a somatotroph responding to adrenaline. Line 3 shows a cell negative to all three antibodies (remainder group) and unresponsive to adrenaline. Using this procedure we were able to analyse the response of typed single cells present into the microscope field.

Figure 5B compares the $[Ca^{2+}]_i$ responses to adrenaline of the different cell types defined by MSPI in a typical experiment. The traces are averaged for all the cells of the same type present in the microscope field. It

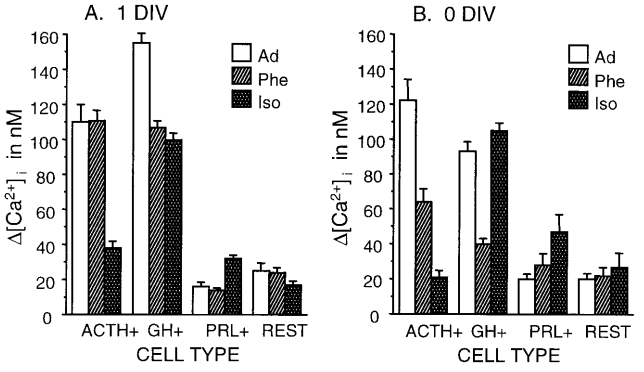


**Fig. 6A, B** Comparison of the effects of adrenergic agonists on the different AP cell types. The figure summarises the results of nine different experiments in cells cultured for 1 day (**A**, *1 DIV*) and 11 experiments with freshly prepared cells (**B**, *0 DIV*). The concentrations of adrenaline, phenylephrine and isoproterenol were as in Fig. 2. The *bars* indicate the mean (±SEM) increase of $[Ca^{2+}]_i$, (nanomoles/l) in the 30 s following stimulation with the agonist. The cells were identified by MSPI as in Fig. 4. Each group included 105–740 cells (between three and six experiments)

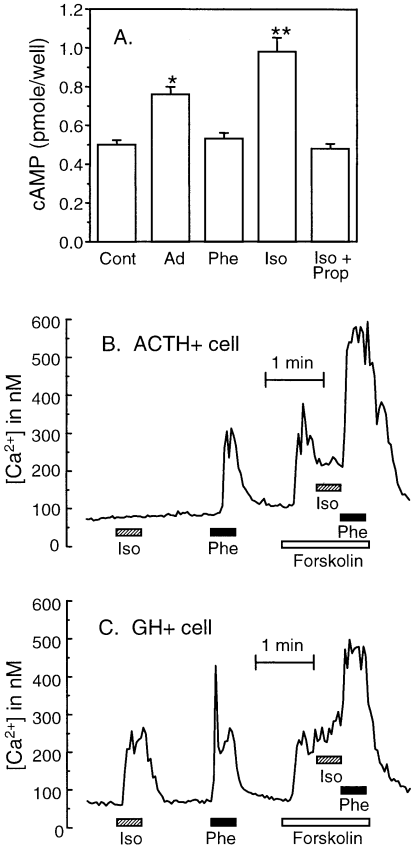


**Fig. 7A–C** Correlation between cAMP and adrenergic action. **A** cAMP levels after 15-min stimulation with different adrenergic agonists (concentrations as in Figs. 2 and 3; see Materials and methods for details). Means±SEM, $n$=18–36. **P*<0.005; ***P*<0.001 vs. control (Student's *t*-test). **B**, **C** Additivity of the effects of adrenergic agonists (adrenaline, phenylephrine, isoproterenol, propranolol) with forskolin (5 μM). Concentrations as in Figs. 2 and 3

seems clear that the responses to adrenaline were very much restricted to two cell types, corticotrophs and somatotrophs, whereas lactotrophs and cells in the remainder group responded poorly or not at all. To examine cell-specific expression of adrenoceptor subtypes, phenylephrine and isoproterenol were used as $\alpha_1$- and β-adrenoceptor agonists, respectively.

Figure 6 summarises the single-cell analysis from 20 similar experiments. Results are shown as $\Delta[Ca^{2+}]_{i,max}$. The mean responses to adrenaline, phenylephrine and isoproterenol are compared for each cell type. In corticotrophs the adrenergic response was mediated mainly by $\alpha_1$-receptors whereas somatotrophs responded well to both $\alpha_1$- and β-agonists (Fig. 6A). In fresh cells the same trends were observed although the responses to the $\alpha_1$-agonist were smaller (Fig. 6B), suggesting that, as reported before [2], cell culture induced expression of $\alpha_1$-receptors. The $\alpha_1$-response, however, still existed in corticotrophs at 0 DIV. Lactotrophs, thyrotrophs and gonadotrophs showed little response to either agonist (Fig. 6A and B). When the results were analysed in terms of fractions of responding cells (responders defined as cells giving a $\Delta[Ca^{2+}]_i$>50 nM) the same trends were seen. Corticotrophs responded preferentially to the $\alpha_1$-agonist (45 and 58% responders at 0 and 1 DIV, respectively) whereas somatotrophs responded to both $\alpha_1$- and β-agonists ($\alpha_1$: 26 and 47% at 0 and 1 DIV, respectively; β: 42 and 61% at 0 and 1 DIV, respectively). The strength of the response to the $\alpha_1$ agonist increased after 24 h in culture. For example, strong responders ($\Delta[Ca^{2+}]_i$>200 nM) increased from 3 to 21% in somatotrophs and from 6 to 22% in corticotrophs.

In many cells $\alpha_1$- and β-receptors coexisted. Of the somatotrophs, 25% showed both $\alpha_1$- and β-responses, 20% only β and 19% only $\alpha_1$ (1910 cells at both 1 and 0 DIV). Of the corticotrophs, most (44%) showed only $\alpha_1$-responses, 15% responded to both and 4% only to β (525 cells at both 1 and 0 DIV).

Typically, β-adrenergic receptors are coupled to adenylate cyclase. Adrenaline and isoproterenol increased cAMP levels in AP cells and the effect of isoproterenol was blocked by propranolol (Fig. 7A). The $\alpha_1$-agonist phenylephrine had no effect on cAMP. To establish whether the increase in $[Ca^{2+}]_i$ elicited by β-adrenergic agents was mediated by cAMP, the additivity of the responses with those to forskolin, a direct activator of adenylate cyclase, was examined. Forskolin increased $[Ca^{2+}]_i$ in both corticotrophs and somatotrophs (Fig. 7B and C) but had only a minor effect in the other AP cell types (not shown). Similar results were obtained with 8-Br-cAMP (500 μM), a membrane-permeant analogue of cAMP (not shown). Forskolin did not prevent a further effect of phenylephrine on $[Ca^{2+}]_i$ (Fig. 7B and C) whereas in cells first treated with forskolin, isoproterenol had no additional effect on $[Ca^{2+}]_i$. This occlusion by forskolin of the effect of the β-adrenergic agonist indicates that the latter's action was in fact mediated by cAMP.

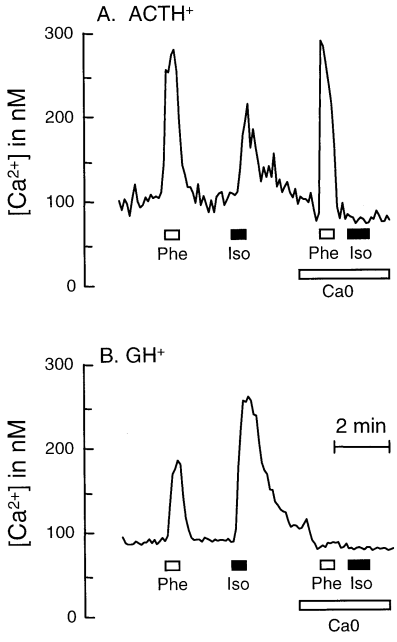


**Fig. 8A, B** $Ca^{2+}$ dependence of the effects of phenylephrine and isoproterenol in corticotrophs and somatotrophs. **A** Average of three cells. **B** Average of 47 cells. The $Ca^{2+}$-free medium contained 0.1 mM EGTA. Other details as in Figs. 2 and 3. Representative of three similar experiments

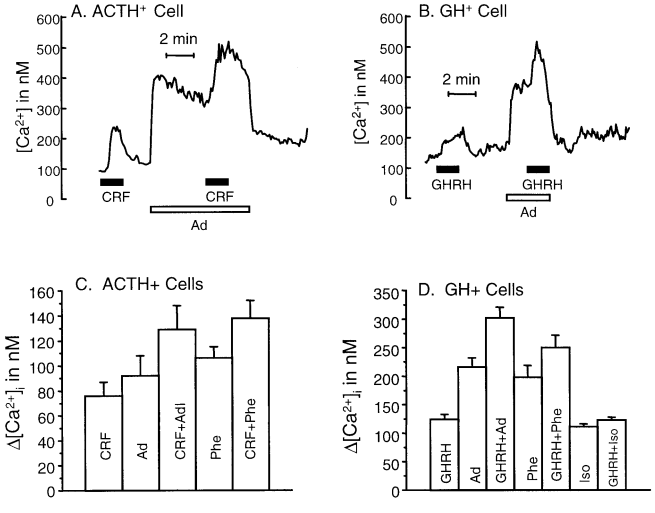


**Fig. 9** Additivity of the effects of hypothalamic releasing factors [corticotropin-releasing factor (*CRF*) and growth hormone-releasing hormone (*GHRH*), each $2\times10^{-8}$ M] and adrenergic agonists. Other details as in Figs. 2 and 3. **A**, **B** Traces obtained in two representative cells. **C**, **D** Mean (±SEM) $\Delta[Ca^{2+}]_i$, calculated as in Fig. 5. **C** Results of between three and seven experiments (24–92 cells). **D** Results of 6–13 experiments (216–462 cells). Concentrations as in Fig. 2

There were also differences among the actions of $\alpha_1$- and β-adrenergic agonists regarding to their dependence on the presence of $Ca^{2+}$ in the extracellular medium. The effect of isoproterenol depended entirely on the presence of external $Ca^{2+}$ both in somatotrophs and corticotrophs (Fig. 8A and B), suggesting that it was due to stimulation of $Ca^{2+}$ entry. In contrast, the effect of phenylephrine in corticotrophs was not prevented by removal of external $Ca^{2+}$ (Fig. 8A), suggesting that the $\alpha_1$-agonist acts by releasing $Ca^{2+}$ from intracellular stores. Unexpectedly, the effect of phenylephrine in somatotrophs also depended entirely on the presence of extracellular $Ca^{2+}$ (Fig. 8B).

To assess the possible physiological significance of adrenergic regulation, interactions with hypothalamic releasing hormones were investigated. Figure 9 summarises the results of experiments designed to investigate the additivity between the effects of the releasing hormones, corticotropin releasing hormone (CRF) and GHRH, and the adrenergic agonists. The effects of adrenaline were additive to those of the corresponding releasing hormone in corticotrophs (Fig. 9A) and somatotrophs (Fig. 9B). When selective agonists were used, the additivity applied, in both cases, to the $\alpha_1$-mediated effects (Fig. 9C and D), but not to the β-mediated effect (Fig. 9D).

## Discussion

Studies on the effects of catecholamines on secretion of AP hormones have produced conflicting results (see Introduction). Interpretation of the results is complicated by expression of different receptor types, sometimes with antagonistic effects. Extrapolation to the in vivo situation is even more difficult because of central effects of catecholamines, which may also affect AP secretion through the hypothalamus. The present results offer a simplified analytical view, as they refer only to direct actions on AP cells and only one of the possible effects, the response of $[Ca^{2+}]_i$. The single-cell analysis adds the power to identify precisely the cell target and to look for the convergence of different mechanisms within the same cell. About one-third of the individual pituitary cells were able to respond to adrenaline with an increase of $[Ca^{2+}]_i$, but responses were not homogeneous within the different cell types. Most of the responding cells could be classified as corticotrophs and somatotrophs by the hormone they stored. Within the other three cell subpopulations, lactotrophs, gonadotrophs and thyrotrophs, there was little response to adrenergic agonists. Results were comparable in cells cultured for 1 day (1 DIV) and in freshly isolated cells (0 DIV), indicating that the response to adrenaline is not an artefact induced by culture, but a genuine property of AP cells.

Both $\alpha_1$- and β-receptors could be identified by the use of specific agonists and specific inhibitors. Individual cells expressing either or both receptors were identified. β-Receptors were present preferentially in somatotrophs whereas $\alpha_1$-receptors were dominant in corticotrophs, although this differential expression was not strict. Expression of $\alpha_1$-responses was strengthened by culture of the cells whereas β-responses were little modified.

Stimulation of β-adrenergic receptors increased cAMP and $[Ca^{2+}]_i$, the latter being dependent entirely on the

presence of external $Ca^{2+}$ and blocked by dihydropyridine antagonists of voltage-gated $Ca^{2+}$ channels. In addition, the increase of $[Ca^{2+}]_i$ was occluded by previous treatment with forskolin, a direct stimulator of adenylate-cyclase. Thus, it seems clear that the β-adrenergic agonists act by stimulating $Ca^{2+}$ entry through voltage-gated channels by a cAMP-mediated mechanism. GHRH acts in somatotrophs by activation of adenylate-cyclase, stimulation of a $Na^+$ current, depolarization and $Ca^{2+}$ entry through voltage-gated channels [29]. The action of the β-adrenergic agonists seems to be the same, and the observation that the effects of isoproterenol and GHRH were not additive (Fig. 9) reinforces this view.

Stimulation of $\alpha_1$-adrenergic receptors produced in corticotrophs an increase of $[Ca^{2+}]_i$ that was independent of the presence of external $Ca^{2+}$ and not occluded by forskolin. These results suggest that $\alpha_1$-receptors are coupled to phospholipase C and inositol trisphosphate-mediated $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores in these cells. This is consistent with the usual coupling mechanisms of $\alpha_1$-receptors and with previous observations in cultured rat somatotrophs [46]. As expected, the effects of phenylephrine and CRF (coupled to adenylate cyclase) were additive (Fig. 9). In somatotrophs, the effect of phenylephrine was dependent on external $Ca^{2+}$ (Fig. 8), suggesting that the $\alpha_1$-agonist acted by stimulating $Ca^{2+}$ entry in these cells. The effect was not occluded by forskolin (Fig. 7C) and was additive with the action of GHRH (Fig. 9D). Thus, the action of phenylephrine in somatotrophs was not consistent with either stimulation of PLC or with adenylate cyclase.

Clonidine, an $\alpha_2$-adrenergic agonist, antagonized the $[Ca^{2+}]_i$-increasing effect of both $\alpha_1$- and β-agonists (Fig. 4). These results are consistent with previous reports in goldfish [22, 52] and ovine [41] pituitary cells, in which $\alpha_2$-adrenoceptor stimulation inhibits both basal and stimulated GH secretion. In goldfish cells clonidine reduces cAMP production, but this is not the only mechanism for inhibition of secretion [52]. The mechanisms involved were not addressed here, but the fact that both the $\alpha_1$- and the β-effects were antagonized suggests complex effects.

It is difficult to explain how such a variety of adrenoceptors, sometimes coexisting in the same cell, should serve and refine the function of the gland. It must be taken into account that other signalling systems may act together with $Ca^{2+}$ to modulate their effects on secretion. In any case, the results shown here suggest that the plasma adrenaline concentrations reached during stress should be adequate to stimulate secretion of GH and ACTH by direct effect on AP cells, thus contributing to the hormonal response to stress, as proposed long ago [23]. The same would be true if noradrenaline were released to the hypophysial portal plasma by hypothalamic neurones, as has been suggested [18]. The β-adrenergic component would act on the same mechanisms as the hypothalamic releasing factors GHRH and CRH, whereas the $\alpha_1$-component would act through a different mechanism, which is additive to the effects of the releasing factors.

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## References

1. Alarcón P, García-Sancho J (2000) Differential calcium responses to the pituitary adenylate cyclase-activating polypeptide (PACAP) in the five main types of anterior pituitary. Pflügers Arch 440:685–691
2. Al-Damluji S (1993) Adrenergic control of the secretion of anterior pituitary hormones. Bailliére's Clin Endocrinol Metab 7:355–392
3. Alvarez J, Montero M, García-Sancho J (1999) Subcellular $Ca^{2+}$ dynamics. News Physiol Sci 14:161–168
4. Aoki Y, Iwasaki Y, Katahira M, Oiso, Y, Saito H (1997) Regulations of the rat proopiomelanocortin gene expression in AtT-20 cells. Effects of the common secretagogues. Endocrinology 138:1923–1927
5. Auteliano DJ, Lundblad JR, Blum M, Roberts JL (1989) Hormonal regulation of POMC gene expression. Annu Rev Physiol 51:715–726
6. Axelrod J, Reisine TD (1984) Stress hormones, their interaction and regulation. Science 224:452–459
7. Baes M, Denef C (1984) $\beta_2$-Receptors in the rat anterior pituitary mediate adrenergic stimulation of prolactin release. Life Sci 34:1447–1454
8. Battaglia G, Shannon M, Titeler M (1983) Initial detection of [3H]prazosin-labeled alpha 1-receptors in the porcine pituitary neurointermediate lobe. Mol Pharmacol 24:409–412
9. Berridge MJ, Lipp P, Bootman MD (2000) The versatility and universality of calcium signalling. Nature Rev 1:11–21
10. Bilezikjian LM, Vale WW (1983) Stimulation of adenosine 3',5' monophosphate production by growth hormone releasing factor and its inhibition by somatostatin in anterior pituitary cells in vitro. Endocrinology 113:1726–1731
11. Chen C, Vincent J-D, Clarke IJ (1994) Ion channels and signal transduction pathways in the regulation of growth hormone secretion. Trends Endocrinol Metab 5:227–233
12. De Souza EB (1985) Beta-2-adrenergic receptors in pituitary. Neuroendocrinology 41:289–296
13. De Souza EB, Kuyatt BL (1987) Alpha-1 adrenergic receptors in the neural lobe of the rat pituitary, autoradiographic identification and localization. Endocrinology 120:2227–2233
14. Denef C, Baes M (1982) β-adrenergic stimulation of prolactin release from superfused pituitary cell aggregates. Endocrinology 111:356–358
15. Farrell GL, McCann SM (1952) Detectable amounts of ACTH hormone in blood following adrenaline. Endocrinology 50: 274–278
16. Giguere V, Labrie F (1983) Additive effects of adrenaline and corticotropin-releasing factor (CRF) on adrenocorticotropin release in rat anterior pituitary cells. Biochem Biophys Res Commun 110:456–462
17. Giguere V, Cote J, Labrie F (1981) Characteristics of $\alpha_1$-adrenergic stimulation of adrenocorticotropin secretion in rat anterior pituitary cells. Endocrinology 109:757–762
18. Johnston CA, Gibbs DM, Negro-Vilar A (1983) High concentrations of adrenaline derived from a central source and of 5-hydroxyindole-3-acetic acid in hypophysial portal plasma. Endocrinology 113:819–821
19. Kamberi IA, Mical RS, Porter JC (1970) Effect of anterior pituitary perfusion and intraventricular injection of catecholamines and indoleamines on LH release. Endocrinology 87: 1–12
20. Krieg RJJr, Thorner MO, Evans WS (1986) Sex differences in β-adrenergic stimulation of growth hormone secretion in vitro. Endocrinology 119:1339–1342
21. Lamberts SW, McLeod RM (1990) Regulation of prolactin secretion at the level of the lactotroph. Physiol Rev 70, 279–318

22. Lee EKY, Chan VCC, Chang, JP, Yunker WK, Wong AOL (2000) Norepinephrine regulation of growth hormone release from goldfish pituitary cells. I. Involvement of $\alpha_2$ adrenoreceptor and interactions with dopamine and salmon gonadotropin-releasing hormone. J Neuroendocrinol 12:311–322
23. Long CNH (1947) The conditions associated with the secretion of adrenal cortex. Fed Proc 6:461–471
24. López MG, Villarroya M, Lara B, Martínez-Sierra R, Albillos A, García AG, Gandía L (1994) Q- and L-type $Ca^{2+}$ channels dominate the control of secretion in bovine chromaffin cells. FEBS Lett 349:331–337
25. Luini A, Lewis D, Guild S, Corda D, Axelrod J (1985) Hormone secretagogues increase cytosolic calcium by increasing cAMP in corticotropin-secreting cells. Proc Natl Acad Sci USA 82:8034–8038
26. Maertens P, Denef C (1987) Alpha-adrenergic stimulation of growth hormone release in perifused rat anterior pituitary reaggregate cell cultures. Mol Cel Endocrinol 54:203–208
27. Mezey E, Reisine TD, Palkovits M, Brownstein MJ, Axelrod J (1983) Direct stimulation of $\beta_2$-adrenergic receptors in rat anterior pituitary induces the release of adrenocorticotropin in vivo. Proc Natl Acad Sci USA 80:6728–6731
28. Mollard P, Schlegel W (1996) Why are endocrine pituitary cells excitable? Trends Endocrinol Metab 7:361–365
29. Naumov AP, Herrington J, Hille B (1994) Actions of growth hormone-releasing hormone on rat pituitary cells: intracellular calcium and ionic currents. Pflügers Arch 427:414–421
30. Pandiella A, Reza Elahi F, Vallar L, Spada A (1988) $\alpha_1$-Adrenergic stimulation of in vitro growth hormone release and cytosolic free $Ca^{2+}$ in rat somatotrophs. Endocrinology 122: 1419–1425
31. Perkins SN, Evans WS, Thorner MO, Cronin MJ (1983) Beta-adrenergic stimulation of GH release from perifused anterior pituitary cells. Neuroendocrinology 37:473–475
32. Perkins SN, Evans WS, Thorner MO, Gibbs DM, Cronin MJ (1985) β-Adrenergic binding and secretory responses of the anterior pituitary. Endocrinology 117:1818–1825
33. Peters JR, Foord SM, Dieguez C, Scanlon MF, Hall R (1983) $\alpha_1$-Adrenoreceptors on intact anterior pituitary cells: correlation with adrenergic stimulation of thyrotropin secretion. Endocrinology 113:133–140
34. Plotsky PM (1985) Hypophyseotropic regulation of adenohypophyseal adrenocorticotropin secretion. Fed Proc 44:207–213
35. Rawlings SR (1996) Pituitary adenylate cyclase-activating polypeptide regulates $[Ca^{2+}]_i$ and electrical activity pituitary cells through cell type-specific mechanisms. Trends Endocrinol Metab 7:374–378
36. Ray KP, Gomm JJ, Law GJ, Sigournay C, Wallis M (1986) Dopamine and somatostatin inhibit forskolin-stimulated prolactin and growth hormone secretion but not stimulated cyclic AMP levels in sheep anterior pituitary cell cultures. Mol Cel Endocrinol 45:175–182
37. Reisine TD, Heisler S, Hook VYH, Axelrod J (1983) Activation of $beta_2$-adrenergic receptors on mouse anterior pituitary tumor cells increases cyclic adenosine 3′,5′-monophosphate synthesis and adrenocorticotropin release. J Neurosci 3:725–732
38. Ritchie AK, Kuryshev YA, Childs GV (1996) Corticotropin-releasing hormone and calcium signaling in corticotropes. Trends Endocrinol Metab 7:365–369
39. Rivier C, Vale W (1983) Modulation of stress induced ACTH release by corticotropin-releasing factor, catecholamines and vasopressin. Nature 305:325–327
40. Sato M, Kubota Y, Malbon CC, Tohyama M (1989) Immunohistochemical evidence that most rat corticotrophs contain beta-adrenergic receptors. Neuroendocrinology 50:577–583
41. Soyoola EO, Burgess MF, Bird RC, Kemppainen RJ, Williams JC, Sartin JL (1994) Neurotransmitter receptor agonists regulate growth hormone gene expression in cultured ovine pituitary cells. Proc Soc Exp Biol Med 207:26–33
42. Stojilkovic SS, Tomic M (1996) GnRH-induced calcium and current oscillations in gonadotrophs. Trends Endocrinol Metab 7:379–384
43. Takahara J, Arimura A, Schally AV (1974) Effect of catecholamines on the TRH-stimulated release of prolactin and growth hormone from sheep pituitaries in vitro. Endocrinology 95:1490–1494
44. Thomas GB, Cummins JT, Smythe GA, Gleeson RM, Dow RC, Fink G, Clarke IJ (1989) Concentrations of dopamine and noradrenaline in hypophysial portal blood in the sheep and rat. J Endocrinol 121:141–147
45. Tilders FJ, Berkenbosch F, Smelik, PG (1982) Adrenergic mechanisms involved in the control of pituitary-adrenal activity in the rat, a β-adrenergic stimulatory mechanism. Endocrinology 110:114–120
46. Tse A, Tse FW (1998) α-Adrenergic stimulation of cytosolic $Ca^{2+}$ oscillations and exocytosis in identified rat corticotrophs. J Physiol (Lond) 512:385–393
47. Vale W, Vaughan J, Smith M, Yamamoto G, Rivier J, Rivier C (1983) Effects of synthetic ovine corticotropin-releasing factor, glucocorticoids, catecholamines, neurohypophysial peptides, and other substances on cultured corticotropic cells. Endocrinology 113:1121–1131
48. Villalobos C, Nuñez L, García-Sancho J (1996) Functional glutamate receptors in a subpopulation of anterior pituitary cells. FASEB J 10:654–660
49. Villalobos C, Alonso-Torre SR, Núñez L, García-Sancho J (1997) Functional ATP receptors in rat anterior pituitary cells. Am J Physiol 273:C1963–C1971
50. Villalobos C, Nuñez L, Frawley LS, García-Sancho J, Sánchez A (1997) Multi-responsiveness of single anterior pituitary cells to hypothalamic releasing hormones: a cellular basis for paradoxical secretion. Proc Natl Acad Sci USA 94:14132–14137
51. Villalobos C, Nuñez L, García-Sancho J (1997) Mechanisms for stimulation of anterior pituitary cells by arginine and other amino acids. J Physiol (Lond) 502:421–431
52. Yunker WK, Lee EKY, Wong AOL, Chang JP (2000) Norepinephrine regulation of growth hormone release from goldfish pituitary cells. II. Intracellular sites of action. J Neuroendocrinol 12:323–333