Requirement for Adenosine Triphosphate for Stimulation *in vitro* of Ox Growth-Hormone Release

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Rotenone, carbonyl cyanide chloromethoxyphenylhydrazone and 2,4-dinitrophenol cause parallel falls in ox pituitary ATP content and growth-hormone release in the presence of 65mM-K⁺. Carbonyl cyanide chloromethoxyphenylhydrazone (0.4 µg/ml) prevented the rise in growth-hormone release, but not the rise in 3':5'-cyclic AMP content after addition of prostaglandin E₂ (1 µg/ml). The rate of hormone release from unstimulated slices and from slices in the presence of Ba²⁺ at 2.3 or 6.9mM was proportional to the ATP content of the slices. Carbonyl cyanide chloromethoxyphenylhydrazone at concentrations that inhibited release in the presence of the three secretagogues did not alter the pituitary contents of Na⁺, K⁺ or Ca²⁺. The implications of these observations are discussed in terms of the mechanism of stimulus-secretion coupling.

The evidence that an increase in cytoplasmic free Ca²⁺ couples stimulation to secretion in the adrenal medulla (Douglas, 1968), and in many other secretory systems (Rubin, 1970), has led to the suggestion that there is a fundamental similarity between stimulus-secretion coupling in secretory tissues and excitation-contraction coupling in muscle. It is not known how far this similarity extends. In muscle Ca²⁺ relieves actomyosin adenosine triphosphatase from inhibition by the troponin complex (Ebashi & Kodama, 1965), but there is no evidence that Ca²⁺ activates an adenosine triphosphatase during secretion. The secretory pathway for growth hormone appears to require ATP at three points. In the rat somatotroph, pulse-labelling experiments have shown that protein transfer to the Golgi apparatus, and granule formation in the Golgi region, both require ATP (Howell & Whitfield, 1973). In the heifer anterior pituitary, ATP is required for the stimulation *in vitro* of growth-hormone release by 65mM-K⁺ (Schofield & Stead, 1971). If one of these ATP-requiring steps is the process that is activated by Ca²⁺ and normally limits the rate of hormone release, then secretagogues that require Ca²⁺, such as prostaglandin E₂ (Cooper *et al.*, 1972), or that mimic the action of Ca²⁺, as does Ba²⁺, should show ATP-dependence. Moreover, ATP should be required at all rates of stimulated release.

In the present paper we report the effect of graded decreases in pituitary ATP, by using mitochondrial poisons, on the stimulation of growth-hormone release by prostaglandin E₂, and two concentrations of Ba²⁺. We have also measured the effect of the

uncoupler carbonyl cyanide chloromethoxyphenylhydrazone on pituitary ion contents and the 3':5'-cyclic AMP response to prostaglandin E₂. The data are consistent with the hypothesis that Ba²⁺, prostaglandin E₂ and 65mM-K⁺ increase the rate of growth-hormone release by acting on an ATP-dependent step.

Materials and Methods

Reagents

[¹⁴C]Sorbitol and [¹²⁵I]iodide were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ox growth hormone was obtained from the Hormone Distribution Officer, National Institutes of Health, Bethesda, Md., U.S.A., and was labelled by using chloramine-T as described by Cooper *et al.* (1972). Prostaglandin E₂ was a gift from Dr. J. E. Pike, of Upjohn Ltd., Kalamazoo, Mich., U.S.A. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

Pituitary slice incubation

Pituitary glands were removed from heifers within 5min of slaughter, the posterior lobe was cut away, and the anterior lobe was sliced with a hand microtome as described by Schofield (1967a). The slices (weighing about 20mg) were incubated at 37°C in 1ml of bicarbonate medium (Krebs & Henseleit, 1932) containing glucose (5.5mM), sodium DL-3-hydroxybutyrate (2.5mM) and sodium glutamate (2.5mM). To minimize basal release the slices were preincubated for 30–60min and were not handled during removal of medium and addition of fresh

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medium to the flask. At the end of the incubation the medium was removed and stored at -15°C before determination of the growth-hormone concentration by double-antibody radioimmunoassay (Schofield, 1967a), and the tissue extracted for measurement of tissue parameters. For determination of intracellular ion contents, tissue slices were lightly blotted and weighed wet. They were then dried for 36h over P_2O_5 in a vacuum desiccator, after which time the weights were constant and the dry weight was measured (Schofield, 1971). The dry slices were then extracted as described below.

Tissue extraction procedures

When ATP was the only parameter measured the tissue was homogenized in 5% (w/v) HClO_4 (0°C) and the insoluble protein removed by centrifugation. In experiments with K^+ as secretagogue, the ATP concentration in neutralized extracts was measured by using glucose 6-phosphate dehydrogenase (Greengard, 1965), and in all other experiments a firefly luciferase assay was used (Ashcroft *et al.*, 1973). When 3':5'-cyclic AMP and ATP were measured in the same extract, the tissue was homogenized in 1.25M- H_2SO_4 , centrifuged, and a portion retained for ATP assay, and the remainder deproteinized by the barium acetate and trisodium phosphate procedure described by McPherson & Schofield (1972). 3':5'-Cyclic AMP was measured by the protein-binding assay described by Cooper *et al.* (1972). When pituitary contents were measured, the tissue was extracted in 2M- HNO_3 , and the ion contents were measured by using an EEL 240 atomic-absorption spectrophotometer (Schofield, 1971). The intracellular ion concentrations were calculated by subtracting the ion content of the extracellular water as measured by the [^{14}C]sorbitol content of each slice (Schofield, 1971).

Results and Discussion

ATP and K^+ -stimulated growth-hormone release

The effect of three mitochondrial poisons on pituitary ATP concentrations, and the stimulation of growth-hormone release by 65mM- K^+ , are shown in Fig. 1. The uncouplers carbonyl cyanide chloromethoxyphenylhydrazone and dinitrophenol, and the respiratory poison rotenone, all caused dose-dependent decreases in pituitary ATP, and parallel decreases in the rate of growth-hormone release. Since these compounds lower the tissue ATP concentrations by different mechanisms, the observation that they have parallel effects on hormone release suggests that they influence release through changes in ATP rather than through other non-specific effects. In these and subsequent experiments the

Fig. 1. Effects of (a) dinitrophenol, (b) carbonyl cyanide chloromethoxyphenylhydrazone and (c) rotenone on pituitary ATP content and growth-hormone release in the presence of 65mM- K^+ .

Pituitary slices were preincubated for 30 min in the presence of dinitrophenol, carbonyl cyanide chloromethoxyphenylhydrazone or rotenone, and then incubated for 60 min again in the presence of dinitrophenol, carbonyl cyanide chloromethoxyphenylhydrazone or rotenone in medium in which the K^+ concentration had been increased to 65 mM, and the Na^+ concentration decreased to 84 mM. The growth-hormone release during this 60 min incubation (—●—) and the pituitary ATP content at the end of the incubation (---●---) are shown; the bars represent S.E.M. for eight slices. The control growth-hormone output by unstimulated slices is also shown (○).

pituitary tissue was preincubated for 30min in the presence of the mitochondrial poisons, and the stimulus was then added at this point. Fig. 2 shows that the major effect of carbonyl cyanide chloromethoxyphenylhydrazone occurs within 30min, and the tissue ATP concentration remains relatively constant during the subsequent 60min incubation.

Fig. 2. Effect of carbonyl cyanide chloromethoxyphenylhydrazone on pituitary ATP content

Pituitary slices were incubated in medium containing glucose, sodium hydroxybutyrate and sodium glutamate (2.5mM), and the tissue ATP concentration was measured after the time shown. Data are means and vertical bars s.e.m. for four to seven slices. ●, Control; ▲, carbonyl cyanide chloromethoxyphenylhydrazone (0.1 μg/ml); ○, carbonyl cyanide chloromethoxyphenylhydrazone (0.4 μg/ml).

ATP and prostaglandin E₂-stimulated growth-hormone release

The rate of any ATP-dependent process will decrease as the tissue ATP concentration falls below the *K_m*(ATP) of that process. Prostaglandin E₂ increases the pituitary concentration of 3':5'-cyclic AMP (Cooper *et al.*, 1972), and the effect of carbonyl cyanide chloromethoxyphenylhydrazone on the increases in 3':5'-cyclic AMP and growth-hormone release induced by prostaglandin E₂ was measured to find out whether a decrease in tissue ATP could decrease the rate of release by an effect on adenylate cyclase. Table 1 shows that carbonyl cyanide chloromethoxyphenylhydrazone (0.4 μg/ml) prevented the rise in growth-hormone release without altering the increase in tissue 3':5'-cyclic AMP, which could indicate that the *K_m*(ATP) of adenylate cyclase is lower than the *K_m*(ATP) of the ATP-dependent step in the secretory process. It should be noted that the pituitary 3':5'-cyclic AMP concentration was measured 10min after prostaglandin addition, at which time the maximum response is observed, whereas growth-hormone release was measured after 60min, at which time the 3':5'-cyclic AMP concentration had fallen to basal values (Cooper *et al.*, 1972). It may also be relevant that in the rat anterior pituitary, Hertelendy *et al.* (1971) found that valinomycin (10 μM) did not prevent the rise in tissue 3':5'-cyclic AMP 30min after addition of theophylline (5mM), but it inhibited growth-hormone release. However, they did not measure the concentration of ATP after valinomycin treatment.

ATP and Ba²⁺-stimulated growth-hormone release

The effect of increasing the extracellular concentration of Ba²⁺ over the range 0–20mM was to increase growth-hormone release to a maximum

Table 1. Effect of carbonyl cyanide chloromethoxyphenylhydrazone on the increases in growth-hormone release and 3':5'-cyclic-AMP concentration induced by prostaglandin E₂

Pituitary slices were preincubated for 30min with or without carbonyl cyanide chloromethoxyphenylhydrazone (CCCP) (0.2 μg/ml), and then incubated for 10 or 60min with or without carbonyl cyanide chloromethoxyphenylhydrazone (0.4 μg/ml) and prostaglandin E₂ (1 μM). The data are given as mean ± s.e.m. for the number of slices shown in parentheses: the slices were obtained from six different pituitary glands.

Tissue parameter	Incubation conditions	Parameter measured at 10min		Parameter measured at 60min	
		In absence of CCCP	In presence of CCCP	In absence of CCCP	In presence of CCCP
ATP content (μmol/g wet wt.)	Control	0.81 ± 0.06 (26)	0.31 ± 0.05 (26)	0.70 ± 0.04 (22)	0.22 ± 0.03 (22)
	Prostaglandin E ₂	0.91 ± 0.07 (26)	0.32 ± 0.05 (26)	0.67 ± 0.05 (22)	0.21 ± 0.03 (22)
3':5'-Cyclic AMP content (nmol/g wet wt.)	Control	0.23 ± 0.05 (23)	0.22 ± 0.04 (23)		
	Prostaglandin E ₂	0.64 ± 0.13 (23)	0.53 ± 0.09 (23)		
Growth-hormone release rate (mg/h per g wet wt.)	Control	0.16 ± 0.02 (20)	0.12 ± 0.02 (20)	0.29 ± 0.03 (20)	0.16 ± 0.03 (20)
	Prostaglandin E ₂	0.23 ± 0.03 (20)	0.15 ± 0.03 (20)	0.66 ± 0.06 (20)	0.23 ± 0.05 (20)

Fig. 3. Effect of Ba²⁺ on growth-hormone release

Pituitary slices were preincubated for 30 min in medium containing glucose (5.5 mM), sodium hydroxybutyrate (2.5 mM) and sodium glutamate (2.5 mM) and in which MgSO₄ was replaced by MgCl₂. They were then incubated for a further 60 min in the sulphate-free medium to which BaCl₂ (100 mM) was added to replace NaCl. Data are means and the bars S.E.M. for 12 slices.

(Fig. 3). To investigate the ATP-dependence of stimulation by Ba²⁺, two groups of experiments were performed, in which the carbonyl cyanide chloromethoxyphenylhydrazone concentration was varied at 6.9 mM-Ba²⁺, or at 2.3 mM-Ba²⁺. The ATP content of each slice was measured (as $\mu\text{mol/g}$ wet wt.) and expressed as a percentage of the mean ATP content of four control slices from the same pituitary incubated in the absence of carbonyl cyanide chloromethoxyphenylhydrazone and Ba²⁺. The rate of growth-hormone release of each slice was also calculated (as $\mu\text{g/h}$ per mg wet wt.) and expressed as a percentage of the mean output by the same control slices. The data were then divided into six or seven roughly equal groups in each of which the ATP content was within a range of 10%, and the mean ATP content and growth-hormone release rates were calculated for each group. This procedure permits direct comparison between the ATP content and the release rate of each slice, and was adopted because the effect of a membrane-soluble compound such as carbonyl cyanide chloromethoxyphenylhydrazone will depend not only on its concentration, but also on the weight of tissue incubated. Fig. 4 shows that as the ATP concentration of the slices decreased, the rate of hormone release decreased proportionally

Fig. 4. Effect of carbonyl cyanide chloromethoxyphenylhydrazone on the stimulation of growth-hormone release by Ba²⁺

Each point represents the mean hormone release, and the bars the S.E.M., for slices whose ATP concentrations were within a 10% range after incubation in carbonyl cyanide chloromethoxyphenylhydrazone at a range of concentrations between 0 and 0.6 $\mu\text{g/ml}$. $\blacktriangle$, Mean output of seven slices in normal, sulphate-free, medium; $\circ$, mean output of 20 slices in sulphate-free medium containing 2.3 mM-Ba²⁺; $\bullet$, mean output of 13 slices incubated in sulphate-free medium containing 6.9 mM-Ba²⁺.

in control tissue and in slices stimulated at two Ba²⁺ concentrations, which were both below the concentration that gave maximum stimulation.

The ATP-dependence of Ba²⁺-stimulated growth-hormone release was confirmed over a range of Ba²⁺ concentration at two ATP concentrations, obtained by adding 0 or 0.2 μg of carbonyl cyanide chloromethoxyphenylhydrazone/ml. Fig. 5(a) shows that increasing the Ba²⁺ concentration to 10 mM caused a 12% fall in ATP content in the absence of carbonyl cyanide chloromethoxyphenylhydrazone; in the presence of carbonyl cyanide chloromethoxyphenylhydrazone the ATP content was decreased by 73% and was not further affected by Ba²⁺. Fig. 5(b) shows that growth-hormone release was proportional to Ba²⁺ concentration at both concentrations of carbonyl cyanide chloromethoxyphenylhydrazone.

ATP and pituitary ion contents

The Na⁺, K⁺ and Ca²⁺ contents of ox pituitary slices are shown in Table 2. Although these concentrations are high, Kraicer & Milligan (1973) have

reported similar concentrations in rat adenohypophysis, after correction for extracellular water, and it is presumed that a large proportion of the ions are bound. In the presence of carbonyl cyanide chloromethoxyphenylhydrazone (0.4 $\mu\text{g/ml}$) the measured

Na^+ content was 30mM higher, and the K^+ content 27mM lower, than in control tissue, but although this could indicate some inhibition of the sodium pump, the differences were not significant. Carbonyl cyanide chloromethoxyphenylhydrazone might be expected to decrease the ability of mitochondria to store Ca^{2+} , but again the difference in Ca^{2+} content was not significant. The relative insensitivity of pituitary ion contents to decreases in ATP is similar to the lymphocyte, where dinitrophenol (0.8mM) has been shown to change the Na^+ and K^+ contents by only 17 and 22mM respectively after 5h (Quissel & Suttie, 1973).

General discussion

The experiments reported here show that the stimulation of growth-hormone release by prostaglandin E_2 , Ba^{2+} and 65mM- K^+ is decreased by agents that cause a parallel fall in ATP concentration. In the presence of 65mM- K^+ , this correlation is seen when mitochondrial ATP production is inhibited either by compounds that increase membrane proton permeability or inhibit the respiratory chain. Moreover, stimulation of rat growth-hormone release by prostaglandin E_1 , theophylline and dibutyryl cyclic AMP is inhibited by valinomycin (Hertelendy *et al.*, 1971), which decreases mitochondrial ATP production by increasing the membrane permeability to K^+ . Since compounds which inhibit mitochondrial ATP production by three different mechanisms, and which would be expected to have different side effects, have the same effects on release, it is reasonable to conclude that the decrease in ATP concentration is related to the decrease in release. Moreover, since stimulation by a number of different secretagogues is affected, the ATP-dependent step appears to be fundamental to the secretory pathway.

A decrease in the tissue concentration of ATP may affect many intracellular reactions, and inhibition of secretion may therefore be a secondary effect,

Fig. 5. Effect of Ba^{2+} on pituitary ATP concentration and growth-hormone release in the presence and absence of carbonyl cyanide chloromethoxyphenylhydrazone

Pituitary slices were preincubated for 30min in sulphate-free media with or without carbonyl cyanide chloromethoxyphenylhydrazone (0.2 $\mu\text{g/ml}$); they were then incubated for 60min in sulphate-free media with or without carbonyl cyanide chloromethoxyphenylhydrazone and Ba^{2+} . Fig. 5(a) gives the final ATP content of slices incubated in the absence (●) or presence (○) of carbonyl cyanide chloromethoxyphenylhydrazone, and Fig. 5(b) gives the growth-hormone release for these slices during the 60min incubation. The bars represent S.E.M. for eight slices.

Table 2. *Effect of carbonyl cyanide chloromethoxyphenylhydrazone on the ion content of ox anterior pituitary slices*

Pituitary slices were preincubated for 40 min with or without carbonyl cyanide chloromethoxyphenylhydrazone, and then incubated for a further 45 min in the presence of [^{14}C]sorbitol (0.5 $\mu\text{Ci/ml}$) again with or without carbonyl cyanide chloromethoxyphenylhydrazone. All media contained glucose (5.5 mM) as sole substrate. Dry weight, intracellular water and ion contents are given as mean $\pm$ S.E.M. for 12 slices; ATP contents measured in other slices are mean $\pm$ S.E.M. for at least 24 slices.

Parameter	Control	0.1 $\mu\text{g/ml}$	0.4 $\mu\text{g/ml}$
Carbonyl cyanide chloromethoxyphenylhydrazone added ...	(Control)		
Dry wt. (mg/mg wet wt.)	0.217 $\pm$ 0.004	0.196 $\pm$ 0.003	0.193 $\pm$ 0.004
Intracellular water ($\mu\text{l/mg}$ wet wt.)	0.367 $\pm$ 0.019	0.386 $\pm$ 0.018	0.394 $\pm$ 0.031
Intracellular Na^+ (mM)	116 $\pm$ 16	101 $\pm$ 13	146 $\pm$ 23
Intracellular K^+ (mM)	202 $\pm$ 19	188 $\pm$ 15	175 $\pm$ 15
Intracellular Ca^{2+} (mM)	23 $\pm$ 2	26 $\pm$ 3	18 $\pm$ 3
ATP content ($\mu\text{mol/g}$ wet wt.)	0.74 $\pm$ 0.03	0.65 $\pm$ 0.05	0.21 $\pm$ 0.03

owing to indirect dependence of the secretory pathway on ATP. It is therefore noteworthy that carbonyl cyanide chloromethoxyphenylhydrazone at concentrations that decrease tissue ATP and inhibit release did not significantly alter the tissue contents of Na^+ , K^+ and Ca^{2+} , nor the rise in cyclic AMP in response to prostaglandin E_2 . Release appears to involve a process that is dependent on ATP over a higher concentration range than for either the sodium pump or adenylate cyclase. It has been suggested that increases in tissue 3':5'-cyclic AMP lead to growth-hormone release (Schofield, 1967b; Steiner *et al.*, 1970; Cooper *et al.*, 1972), and although this suggestion has been questioned (Peake *et al.*, 1972, 1973) it is clear that the ATP-dependence does not reflect the substrate requirement for adenylate cyclase.

An alternative explanation is that a decrease in the ATP concentration decreases release by inhibition of hormone synthesis. Although carbonyl cyanide chloromethoxyphenylhydrazone has indeed been shown to inhibit rat growth-hormone synthesis (Howell & Whitfield, 1973), this is unlikely to be the only effect of the uncoupler, since in the heifer system it completely abolished (at 0.4 $\mu\text{g/ml}$) the stimulation of release caused by Ba^{2+} (6 mM), prostaglandin E_2 (1 μM) and K^+ (65 mM). Stachura *et al.* (1972) have shown that K^+ (21 mM) causes a fourfold increase in the release of growth hormone synthesized and stored in the 3 h before addition of the secretagogue. Thus carbonyl cyanide chloromethoxyphenylhydrazone must inhibit the increase in release of stored hormone as well as newly synthesized hormone, and it is probable that ATP is required both for synthesis of the hormone and for secretion coupling.

Whether the ATP-dependence results from direct involvement of ATP in the secretory pathway, possibly through a protein kinase or an ATPase, or from some less direct involvement, it would be of some interest to establish that it represents the site of action of secretagogues. The data presented here

show that the rate of growth-hormone release falls in parallel with the ATP concentration in the presence of Ba^{2+} at concentrations too low to give maximal stimulation, and that there is no threshold ATP concentration above which stimulation is independent of ATP. This would be expected if Ba^{2+} increased the rate of the ATP-dependent process. It may also be relevant that stimulation of release by the thiol reagent *p*-chloromercuribenzoate is not inhibited by depletion of pituitary ATP or Ca^{2+} contents (Schofield, 1971), suggesting that ATP and Ca^{2+} are involved in a reaction modified or replaced on the addition of the thiol reagent. It should, however, be noted that the linear relationship observed between ATP content and hormone release could arise if, as the carbonyl cyanide chloromethoxyphenylhydrazone concentration increases, an increasing proportion of the cells have their ATP concentration decreased below that required for initiation of release, whereas the other cells are not affected. Moreover, although carbonyl cyanide chloromethoxyphenylhydrazone, rotenone and dinitrophenol would not be expected to be selective in their effects on different pituitary cell types, there is no evidence that the concentration of ATP measured here is the ATP concentration of the somatographs, which, in the female hamster at least, comprise only 32% of the tissue (Dekker, 1968). It is therefore not established that the rate of growth-hormone release from a given cell is proportional to the ATP content of that cell.

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