Increase in Pituitary Adenosine 3':5'-Cyclic Monophosphate Content and Potentiation of Growth-Hormone Release from Heifer Anterior Pituitary Slices Incubated in the Presence of 3-Isobutyl-1-methylxanthine

By J. GEORGE SCHOFIELD and MARGARET McPHERSON*
Department of Biochemistry, University of Bristol, Bristol BS8 1TD, U.K.

(Received 18 February 1974)

The release of growth hormone from heifer anterior pituitary slices and the cyclic AMP content of the slices were increased by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, both increases being related to inhibitor concentration over the range 0.1–1.0 mM. Neither Ba^{2+} (6.9 or 2.3 mM), K^+ (72 mM), nor *p*-chloromercuribenzoate (20 μM) had any effect on pituitary cyclic AMP content over a 20 min period. 3-Isobutyl-1-methylxanthine potentiated the release of growth hormone in response to Ba^{2+} (2.3 mM) and K^+ (24 mM), but the degree of potentiation did not depend on inhibitor concentration in the same way as did tissue cyclic AMP content. 3-Isobutyl-1-methylxanthine decreased the concentration of K^+ required to give maximum stimulation of growth-hormone release, but did not significantly increase the maximum response to Ba^{2+} . Growth-hormone release in the presence of prostaglandin E_2 (1 μM) was increased by 3-isobutyl-1-methylxanthine and was inhibited by the prostaglandin antagonist, 7-oxa-13-prostynoic acid, although this antagonist increased the pituitary cyclic AMP concentration and potentiated the prostaglandin E_2 -induced rise in cyclic AMP content. The stimulation of growth-hormone release by *p*-chloromercuribenzoate was not potentiated by 3-isobutyl-1-methylxanthine. The data suggest that Ba^{2+} and K^+ act at the same point in the secretory process as 3-isobutyl-1-methylxanthine, although by a different mechanism, and that *p*-chloromercuribenzoate has a different point of action.

The release of growth hormone *in vitro* is increased by substances which increase the pituitary concentration of cyclic AMP. Thus the phosphodiesterase inhibitor theophylline increases the release of bovine (Schofield, 1967*b*) and rat (Hertelendy *et al.*, 1971) growth hormone; prostaglandin E_1 increases the release of rat growth hormone (MacLeod & Lehmeyer, 1970), and prostaglandin E_2 increases the release of bovine growth hormone (Cooper *et al.*, 1972). The release of bovine growth hormone is also stimulated by Ba^{2+} and by high external K^+ (Schofield & Cole, 1971) and by *p*-chloromercuribenzoate (Schofield, 1971). High external K^+ concentrations do not increase the rate of cyclic AMP formation in the rat pituitary (Zor *et al.*, 1970), but the effect of Ba^{2+} and *p*-chloromercuribenzoate on pituitary cyclic AMP content is not known. Thus it is possible that substances which stimulate growth-hormone release (hereinafter referred to as secretagogues) can be placed in two categories depending on their effect on tissue cyclic AMP concentrations, and that these two types of secretagogue either stimulate parallel secretory pathways or the

same pathway through different mechanisms. The present study has investigated the effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, which increases the cyclic AMP content and insulin release in rat islets of Langerhans (Cook & Montague, 1971), on growth-hormone release in response to Ba^{2+} , K^+ and *p*-chloromercuribenzoate. The effects of a range of inhibitor concentrations on pituitary cyclic AMP content and on the secretory response to low concentrations of secretagogues were measured to determine whether the inhibitor potentiates release to the same extent that it increases cyclic AMP content. To see if the maximum secretory responses were altered, 3-isobutyl-1-methylxanthine was added in the presence of a range of secretagogue concentrations.

Materials and Methods

Reagents

Cyclic [2- ^3H]AMP (>20 Ci/mmol) and sodium [^{125}I]iodide (carrier-free, 80–140 mCi/ml) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Ox growth hormone was obtained from

* Present address: Department of Anatomy, University of Bristol, Bristol BS8 1TD, U.K.

the Hormone Distribution Officer, National Institutes of Health, Bethesda, Md., U.S.A., and was iodinated by using chloramine-T as described elsewhere (Cooper *et al.*, 1972). 3-Isobutyl-1-methylxanthine (SC 2964) was a gift from G. D. Searle Co., Chicago, Ill., U.S.A. Prostaglandin E₂ was given by Dr. J. E. Pike of Upjohn Ltd., Kalamazoo, Mich., U.S.A. 7-Oxa-13-prostynoic acid was given by Dr. J. Fried, of Chicago University, U.S.A.

Pituitary incubations

Pituitary glands were removed from heifers within 5 min of slaughter; the posterior lobe was removed and the anterior lobe sliced (Schofield, 1967a). The slices were incubated at 37°C in bicarbonate-buffered medium (Krebs & Henseleit, 1932), containing glucose (5.5 mM), sodium DL-β-hydroxybutyrate (2.5 mM) and sodium glutamate (2.5 mM), and equilibrated with O₂+CO₂ (95:5). The slices, weighing about 20 mg, were normally preincubated for 45 or 60 min in 1.5 ml of medium, which was changed two or three times during this time, before addition of secretagogue. When K⁺ was used as secretagogue, some NaCl was replaced by KCl to give the final K⁺ concentrations stated in the text or the Figures: when BaCl₂ was added, the medium contained MgCl₂ in place of MgSO₄. The preparation of prostaglandin and *p*-chloromercuribenzoate solutions has been described elsewhere (Schofield, 1971; Cooper *et al.*, 1972).

Measurement of cyclic AMP and growth-hormone release

At the end of the incubation in the presence of the stimulus, the media were removed and stored at -15°C before determination of the growth-hormone concentration by radioimmunoassay (Schofield, 1967a). The tissue was extracted in 1.25 M-H₂SO₄, the extract deproteinized by addition of barium

acetate and Na₃PO₄ (McPherson & Schofield, 1972), and the cyclic AMP concentration in the extract was measured by using the protein-binding assay described previously (Cooper *et al.*, 1972).

Results

Effect of 3-isobutyl-1-methylxanthine on growth-hormone release and pituitary cyclic AMP concentration

Table 1 shows that the cyclic AMP content of pituitary slices was increased after 10 min incubation in the presence of 3-isobutyl-1-methylxanthine, and remained elevated after 60 min incubation. The release of growth hormone during this 60 min incubation was doubled by 3-isobutyl-1-methylxanthine. The rise in cyclic AMP content after 10 min and the rise in growth-hormone release over 60 min showed similar dependence on the concentration of 3-isobutyl-1-methylxanthine over the range 0.1–1.0 mM (Fig. 1). Since the tissue cyclic AMP content was related to the concentration of 3-isobutyl-1-methylxanthine and remained constant over a 60 min incubation, it was possible to use the inhibitor to investigate the correlation between increased pituitary cyclic AMP content and the modification of growth-hormone release in response to other secretagogues.

Effect of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of Ba²⁺

Table 2 shows that Ba²⁺ (2.3 or 6.9 mM) had no effect on the pituitary content of cyclic AMP measured at 10 and 30 min: the rise in cyclic AMP observed over this period in the control and stimulated slices was unusual, but no explanation is apparent. The effect of increasing concentrations of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of 2.3 mM-Ba²⁺ is shown in Fig. 2(a). The addition of Ba²⁺ alone slightly increased

Table 1. *Effects of 3-isobutyl-1-methylxanthine and prostaglandin E₂ on tissue cyclic AMP content and growth-hormone release*

Data are means ± S.E.M. for eight slices derived from two pituitaries, preincubated for 45 min in 1.5 ml of medium containing glucose (5.5 mM) and sodium DL-β-hydroxybutyrate (2.5 mM), and then incubated for 10 or 60 min in 1 ml of this medium containing prostaglandin E₂ or 3-isobutyl-1-methylxanthine. The significance of the differences was obtained by using Student's *t* test; **P* < 0.001 for difference from control; †*P* < 0.01 for difference from cyclic AMP content after 10 min in prostaglandin E₂.

Incubation conditions	Growth hormone released (μg/h per mg wet wt.)	Cyclic AMP content (nmol/g wet wt.)	
		After 10 min	After 60 min
Control	0.45 ± 0.08	0.12 ± 0.03	0.06 ± 0.02
Prostaglandin E ₂ (1 μM)	1.00 ± 0.09*	1.45 ± 0.16	0.27 ± 0.06†
3-Isobutyl-1-methylxanthine (1 mM)	1.13 ± 0.12*	1.57 ± 0.22	1.85 ± 0.32
Prostaglandin E ₂ (1 μM) + 3-isobutyl-1-methylxanthine (1 mM)	2.34 ± 0.21	—	—

the rate of release, and addition of 0.1 or 0.25mm-3-isobutyl-1-methylxanthine in the presence of Ba²⁺ markedly enhanced the output of growth hormone: addition of 1mm-3-isobutyl-1-methylxanthine in the presence of Ba²⁺ gave a lower enhancement of release. The effect of 3-isobutyl-1-methylxanthine on the maximum secretory response to Ba²⁺ is shown in Fig. 2(b). At higher concentrations of Ba²⁺ the release of growth hormone tended to a plateau of about 9µg/h per mg wet wt. (approx. 30% of the stored hormone): at these higher Ba²⁺ concentrations the addition of 1mm-3-isobutyl-1-methylxanthine had no significant effect on growth-hormone release.

Effect of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of increased extracellular K⁺

An increase in the concentration of extracellular K⁺ to 72mm had no effect on the pituitary content of cyclic AMP over a 20min period (Table 2), the heifer pituitary behaving in this respect as did the rat pituitary (Zor *et al.*, 1970). The effect of increasing concentrations of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of 24mm-K⁺ is shown in Fig. 3(a). The addition of 0.1 or 0.25mm-3-isobutyl-1-methylxanthine potentiated the stimulation of release by K⁺, and the augmentation of release was less in the presence of 1mm-3-isobutyl-1-methylxanthine. In the presence of 1mm-3-isobutyl-1-methylxanthine the secretory response to K⁺ was maximal at 48mm-K⁺ (Fig. 3b); the secretory response to K⁺ alone passes through a maximum at about 70mm (Schofield & Cole, 1971). The decreased secretory response to higher K⁺ concentrations made it impossible to determine whether 3-isobutyl-1-methylxanthine increased the maximal response to K⁺ or decreased the K⁺ concentration at which it was observed.

Effect of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of p-chloromercuribenzoate

In a single experiment when the pituitary cyclic AMP content was measured over a 20min period in the presence of 20µM-p-chloromercuribenzoate, no change in cyclic AMP content was observed (control 0.12±0.01, cf. p-chloromercuribenzoate-treated 0.15±0.01nmol of cyclic AMP/g wet wt.; n = 4). In the presence of 10µM-p-chloromercuribenzoate, addition of 0.1–1.0mm-3-isobutyl-1-methylxanthine appeared to have an additive rather than a potentiating effect

Fig. 1. Effect of increasing concentrations of 3-isobutyl-1-methylxanthine on pituitary cyclic AMP content and growth-hormone release

Data show the release of growth hormone over 60 min [■; each point is mean ±S.E.M. (vertical bar) for all 24 slices from the six pituitary glands incubated in the presence of 3-isobutyl-1-methylxanthine alone and shown in Figs. 2(a), 3(a) and 4(a) in groups of eight slices] and cyclic AMP content after 10 min (●; each point is mean ±S.E.M. of eight slices from two glands).

Table 2. Effects of increased K⁺ concentration or Ba²⁺ on tissue cyclic AMP content

Slices were preincubated for 45 min in 1.5ml of medium containing glucose (5.5mm) and sodium DL-β-hydroxybutyrate (2.5mm), and then incubated for the time shown in 1 ml of medium. The data are means±S.E.M.; numbers in parentheses denote the number of slices used for each condition.

Condition	Tissue cyclic AMP content (nmol/mg wet wt.) after:				
	5min	10min	15min	20min	30min
Control	0.12±0.02 (4)	0.11±0.03 (4)	0.13±0.04 (4)	0.08±0.01 (4)	—
K ⁺ (72mm)	0.11±0.03 (4)	0.09±0.03 (4)	0.12±0.05 (4)	0.10±0.10 (4)	—
Control	—	0.10±0.02 (8)	—	—	0.25±0.06 (8)
Ba ²⁺ (2.3mm)	—	0.10±0.01 (8)	—	—	0.39±0.08 (8)
Ba ²⁺ (6.9mm)	—	0.09±0.02 (8)	—	—	0.29±0.08 (8)

Fig. 2. Effect of 3-isobutyl-1-methylxanthine in the presence of Ba^{2+}

(a) Data show growth-hormone release in the presence of increasing concentrations of 3-isobutyl-1-methylxanthine in the presence (■) or absence (●) of 2.3 mM- BaCl_2 . Each point is mean $\pm$ s.e.m. (vertical bar) of eight slices taken from the same two pituitary glands. (b) Data show growth-hormone release in the presence of increasing concentrations of BaCl_2 in the presence (■) or absence (●) of 1 mM-3-isobutyl-1-methylxanthine. Each point is mean $\pm$ s.e.m. (vertical bar) for the number of observations shown in parentheses. At each given BaCl_2 concentration the same number of slices derived from the same pituitaries were incubated with and without 3-isobutyl-1-methylxanthine.

Fig. 3. Effect of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of increased intracellular K^+

(a) Data show the effect of increasing concentrations of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of 24 mM- K^+ (■) or 5.9 mM- K^+ (●). Each point is the mean $\pm$ s.e.m. (vertical bar) of eight slices obtained from the same two pituitary glands. (b) Data show the effect of increasing KCl concentrations on growth-hormone release in the presence (■) or absence (●) of 3-isobutyl-1-methylxanthine (1 mM). Each point is mean $\pm$ s.e.m. (vertical bar) for the number of observations shown in parentheses; at each KCl concentration the same number of slices derived from the same pituitary glands was incubated with or without 3-isobutyl-1-methylxanthine.

(Fig. 4a). When the concentration of *p*-chloromercuribenzoate was increased to 20 µM in the presence of 1 mM-3-isobutyl-1-methylxanthine, the increment in release was not observed (Fig. 4b). Thus there is no evidence that *p*-chloromercuribenzoate stimulates a secretory pathway which is modified under conditions of increased cyclic AMP.

Effect of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of prostaglandin E_2

Table 1 shows that prostaglandin E_2 (1 µM) gave identical increases in growth-hormone release over 60 min, and in cyclic AMP content at 10 min, as did 3-isobutyl-1-methylxanthine (1 mM). However, the cyclic AMP concentration, which remained constant in the presence of 3-isobutyl-1-methylxanthine, as

would be expected if the activity of phosphodiesterase was decreased, fell over a period of 60 min in the presence of prostaglandin E_2 . When prostaglandin E_2 was added in the presence of 3-isobutyl-1-methylxanthine the increase in growth-hormone release (1.89 µg/h per mg) was slightly greater than the sum of the increases in release caused by the two secretagogues individually (1.23 µg/h per mg).

The relationship between the increases in cyclic AMP content and growth-hormone release was further investigated by using the prostaglandin antagonist 7-oxa-13-prostynoic acid. This antagonist alone increased the pituitary cyclic AMP content but not the release of growth hormone, and in the presence of prostaglandin E_2 it potentiated the rise in

cyclic AMP content while blocking the rise in growth-hormone release (Table 3). Thus the antagonist appeared to act as a phosphodiesterase inhibitor, but dissociated the rise in cyclic AMP from a rise in growth-hormone release, as did removal of Ca^{2+} (Cooper *et al.*, 1972). In the rat pituitary, Ratner *et al.* (1973) did not observe this dissociation, the same concentration of 7-oxa-13-prostynoic acid blocking both the secretory and cyclic AMP responses to prostaglandin E_1 . The discrepancy may arise from the use of different prostaglandins, different sampling times [15 min after prostaglandin addition in the present study, as compared with 60 min in the report by Ratner *et al.* (1973)], or from different sensitivities of heifer and rat pituitaries to the antagonist. 7-Oxa-13-prostynoic acid has been shown to antagonize the action of prostaglandin E_2 in gastrointestinal muscle from guinea pig, but not from rat or man (Bennet & Posner, 1971).

Discussion

The data shown that 3-isobutyl-1-methylxanthine increases the release of bovine growth hormone and the pituitary cyclic AMP content with a similar concentration dependence. However, it does not follow that the rise in cyclic AMP content initiates the rise in growth-hormone release. Peake *et al.* (1973) have reported that pure growth-hormone-releasing factor increased pituitary cyclic GMP but not cyclic AMP contents. Moreover the phosphodiesterase inhibitor aminophylline increased the cyclic GMP content of rat pituitaries 13-fold, whereas it increased the cyclic AMP content only threefold (Peake *et al.*, 1972). Thus it is possible that the effects of 3-isobutyl-1-methylxanthine reported here are caused by an increase in pituitary cyclic GMP concentration. The effects of prostaglandin E_2 on guanylate cyclase in the heifer pituitary are not known, but the modification of the responses to

Fig. 4. Effect of 3-isobutyl-1-methylxanthine on growth-hormone release in the presence of *p*-chloromercuribenzoate

(a) Data show the effect of increasing concentrations of 3-isobutyl-1-methylxanthine in the presence (■) or absence (●) of *p*-chloromercuribenzoate (10 μM). Each point is the mean $\pm$ S.E.M. (vertical bar) for eight slices derived from the same two pituitary glands. (b) Data show the effect of increasing concentrations of *p*-chloromercuribenzoate on growth-hormone release in the presence (■) or absence (●) of 3-isobutyl-1-methylxanthine (1 mM). Each point is the mean $\pm$ S.E.M. (vertical bar) for the number of slices shown in parentheses; at each *p*-chloromercuribenzoate concentration the same number of slices derived from the same pituitary glands were incubated with and without 3-isobutyl-1-methylxanthine.

Table 3. Effects of 7-oxa-13-prostynoic acid on prostaglandin E_2 stimulation of growth-hormone release and tissue cyclic AMP content

Data are means $\pm$ S.E.M. for ten slices from four pituitary glands, preincubated for 60 min in 1.5 ml of medium containing glucose (5.5 mM) and sodium DL-β-hydroxybutyrate (2.5 mM), and then incubated for the time shown under the conditions stated. In those flasks containing prostaglandin E_2 and prostynoic acid, the latter was added 30 s before the former. * $P < 0.05$ for difference from control.

Conditions	Cyclic AMP content (nmol/mg wet wt.) after 15 min	Growth hormone released (μg/mg wet wt.) after	
		15 min	60 min
Control	0.09 $\pm$ 0.02	0.67 $\pm$ 0.17	0.99 $\pm$ 0.17
Prostaglandin E_2 (1 μM)	0.26 $\pm$ 0.06*	0.77 $\pm$ 0.28	1.96 $\pm$ 0.42*
7-Oxa-13-prostynoic acid (50 μg/ml)	0.24 $\pm$ 0.06*	0.57 $\pm$ 0.18	1.14 $\pm$ 0.21
Prostaglandin E_2 (1 μM) and 7-oxa-13-prostynoic acid (50 μg/ml)	0.73 $\pm$ 0.18*	0.77 $\pm$ 0.17	0.97 $\pm$ 0.20

prostaglandin E_2 by prostanoic acid is difficult to explain in terms of initiation of secretion by cyclic AMP.

The data show that growth-hormone secretagogues fall into two groups. One group, which includes theophylline (Steiner *et al.*, 1970), aminophylline (Peake *et al.*, 1972), prostaglandin E_2 (Cooper *et al.*, 1972) and 3-isobutyl-1-methylxanthine, also increases pituitary cyclic AMP content. The other group, including Ba^{2+} , K^+ and *p*-chloromercuribenzoate, has no effect on cyclic AMP concentration. 3-Isobutyl-1-methylxanthine at 0.1 and 0.25 mM markedly potentiates the effects of Ba^{2+} or K^+ at concentrations below those needed for maximal stimulation of release. Potentiation was less at 1 mM 3-isobutyl-1-methylxanthine and therefore did not parallel the rise in cyclic AMP content with increasing concentrations of inhibitor. At higher concentrations of Ba^{2+} , 3-isobutyl-1-methylxanthine failed to increase release significantly, which suggests that the maximal secretory response is not increased. These observations are consistent with the hypothesis that Ba^{2+} and K^+ act on the same secretory step as 3-isobutyl-1-methylxanthine. A possible analogy would be with the muscle phosphorylase system, where the kinase may be activated either by increased Ca^{2+} concentrations or by a cyclic AMP-dependent kinase in the presence of an unaltered Ca^{2+} concentration (Brostrom *et al.*, 1971). Both Macleod & Lehmyer (1970) and Hertelendy (1971) observed that prostaglandin E_1 gave a less than additive increment in rat growth-hormone release in the presence of high K^+ , although this might be due to the transient nature of the prostaglandin E_1 -induced changes in cyclic AMP.

No evidence was obtained to suggest that 3-isobutyl-1-methylxanthine potentiated growth-hormone release in response to *p*-chloromercuribenzoate, and stimulation by this secretagogue appears to be independent of cyclic AMP as well as ATP and Ca^{2+} (Schofield, 1971). It would be interesting to determine whether *p*-chloromercuribenzoate increases the frequency of exocytotic images in the electron microscope, since the effect of this secretagogue is

inhibited by Ni^{2+} as are the effects of K^+ , Ba^{2+} and prostaglandin E_2 (Dormer *et al.*, 1974).

We thank Mr. Alan Kerbey for expert assistance. The cost of these investigations was met in part by the British Diabetes Association, the Medical Research Council, and the British Insulin Manufacturers.

References

- Bennet, A. & Posner, J. (1971) *Brit. J. Pharmacol.* **42**, 584–594
- Brostrom, C. O., Hunkeler, F. L. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1961–1967
- Cook, J. R. & Montague, W. (1971) *Biochem. J.* **122**, 115–120
- Cooper, R. H., McPherson, M. & Schofield, J. G. (1972) *Biochem. J.* **127**, 143–154
- Dormer, R. L., Kerbey, A. L., McPherson, M., Manley, S., Ashcroft, S. J. A., Schofield, J. G. & Randle, P. J. (1974) *Biochem. J.* **140**, 135–142
- Hertelendy, F. (1971) *Acta Endocrinol. (Copenhagen)* **68**, 355–362
- Hertelendy, F., Todd, H., Peake, G. T., Machlin, L. J., Johnson, G. & Pounds, G. (1971) *Endocrinology* **89**, 1256–1262
- Krebs, H. A. & Henseleit, A. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–41
- Macleod, R. M. & Lehmyer, J. E. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 1172–1179
- McPherson, M. & Schofield, J. G. (1972) *FEBS Lett.* **24**, 45–48
- Peake, G. T., Steiner, A. L. & Daughaday, W. H. (1972) *Endocrinology* **90**, 212–216
- Peake, G. T., Wilson, M., Steiner, A. L. & Dhariwal, A. P. S. (1973) *Metabolism* **22**, 769–772
- Ratner, A., Wilson, M. C. & Peake, G. T. (1973) *Prostaglandins* **3**, 413–419
- Schofield, J. G. (1967a) *Biochem. J.* **103**, 331–341
- Schofield, J. G. (1967b) *Nature (London)* **215**, 1382–1383
- Schofield, J. G. (1971) *Biochim. Biophys. Acta* **252**, 516–525
- Schofield, J. G. & Cole, E. N. (1971) *Mem. Soc. Endocrinol.* **19**, 185–199
- Steiner, A. L., Peake, G. T., Utiger, R. D., Karl, I. E. & Kipnis, D. M. (1970) *Endocrinology* **86**, 134–136
- Zor, U., Kaneto, T., Schneider, P. G., McCann, S. M. & Field, J. B. (1970) *J. Biol. Chem.* **245**, 2883–2888