Role of Ca^{2+} and Cyclic Nucleotides in the Control of Prostaglandin E Production in the Rat Anterior Pituitary Gland

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In an attempt to relate changes in the intracellular concentration of prostaglandin E to the secretion process, two agents known to increase cyclic nucleotide concentrations and hormone release were added to dispersed rat anterior pituitary cells. They caused increases in the intracellular prostaglandin E concentrations. Increasing the K^+ concentration in the medium (which stimulates hormone release) caused a rapid rise in prostaglandin E concentrations. The addition of the Ca^{2+} ionophore A23187 had a similar effect. The effects of changes in the K^+ and Ca^{2+} concentrations and the addition of EDTA were measured on the redistribution of radioactivity in pituitary glands prelabelled with [^3H]arachidonic acid. Elevated K^+ concentrations stimulated the transfer of label to prostaglandins and free arachidonic acid, suggesting an increased phospholipase A activity. On the other hand, the absence of extracellular CaCl_2 and the addition of EDTA had the opposite effect, which could be cancelled by the addition of sufficient amounts of CaCl_2 . It is concluded that the addition of agents that increase membrane permeability to bivalent cations probably results in an influx of Ca^{2+} and this appears to result in increased phospholipase A activity, which in turn leads to an increase in prostaglandin production.

Cyclic nucleotides have been implicated in the control of the synthesis and release of anterior pituitary hormones (Labrie *et al.*, 1976), and changes in the ionic environment have also been shown to affect the release of these hormones *in vitro* (Hertelendy *et al.*, 1971; Gautvik & Tashjian, 1973; Labella *et al.*, 1973). Ionophores such as A23187 which increase membrane permeability to Ca^{2+} (Scarpa & Inesi, 1972) have been shown to increase the release of insulin and glucagon from the islets of Langerhans (Ashby & Speake, 1975), release of histamine from mast cells (Foreman *et al.*, 1973) and release of somatotropin (growth hormone) from anterior pituitary tissue (Bicknell & Schofield, 1976). Recent work has shown that the concentration of prostaglandin E in rat anterior pituitary cells may also be important in the control of the production of somatotropin (Betteridge & Wallis, 1977, 1979) and lutropin (Barden & Betteridge, 1977). Prostaglandin E synthesis in these cells is increased when precursor concentrations are elevated by stimulating their release from storage in membrane phospholipid (Betteridge & Wallis, 1978).

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It was the purpose of these studies to investigate the relationship between cyclic nucleotides, prostaglandins and changes in membrane permeability to Ca^{2+} caused by changes in extracellular ion concentrations and by the ionophore A23187.

Materials and Methods

Materials

[5,6,8,11,12,14,15- ^3H]Prostaglandin E_2 (150 Ci/mmol) and [5,6,8,9,11,12,14,15- ^3H]arachidonic acid (135 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Tissue-culture media, sera and additions were from Gibco, New York, NY, U.S.A. Synthetic prostaglandins were provided by Upjohn Pharmaceuticals, Crawley, Sussex, U.K. Phospholipid standards were from Lipid Products, Redhill, Surrey, U.K., and standard arachidonic acid, isobutylmethylxanthine and 8-bromo cyclic AMP were from Sigma Chemical Co., Ionophore A23187 was provided by Eli Lilly and Co., Indianapolis, IN, U.S.A. Rat somatotropin (growth hormone) (NIAMDD rat GH-B-3 and GH-RP-1) and monkey anti-(rat somatotropin) serum (NIAMDD anti-rat

GH-serum-1) were gifts from the Rat Pituitary Hormone Program of the National Institute of Arthritis, Metabolism and Digestive Diseases, NIH, Bethesda, MD, U.S.A.

Measurement of prostaglandin E concentrations

Dispersed anterior pituitary cell preparations were obtained from male Sprague-Dawley rats by the method of Vale *et al.* (1972). The cell suspension was washed several times, by centrifugation, with Dulbecco's modified Eagle's medium and plated out in this medium containing 10% (v/v) horse serum, 2.5% (v/v) foetal calf serum, non-essential amino acids and antibiotics (Vale *et al.*, 1972). Three dishes containing dispersed cells were prepared from each pituitary used. The dishes of cells were incubated at 37°C for 4 days and then washed with serum-free medium to remove unattached cells. The cells were preincubated for 1 h with Dulbecco's modified Eagle's medium and then incubated with the experimental media.

After incubation, the medium was removed and the cells were scraped off the dishes into 1 ml of ethanol/0.9% NaCl (1:1, v/v) and then were sonicated for 5 s at setting 1 (Dawe Soniprobe). The prostaglandins were extracted with 2 ml of ethyl acetate. This was then dried *in vacuo* and the extract was treated for use in the radioimmunoassay as described (Barden & Betteridge, 1977).

The antibody used gave less than 1% cross-reaction to prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ and less than 8% cross-reaction to prostaglandins A_1 and A_2 . It did not discriminate sufficiently between prostaglandins E_1 and E_2 , so the results are expressed in terms of prostaglandin E (E_1 and E_2 combined).

Measurement of hormone release

Where the rate of hormone release was to be measured, the media were kept after the experiments outlined above. Samples of the media were diluted (between 1:5 and 1:25) and assayed for somatotropin content with a radioimmunoassay kit obtained from the National Institute of Arthritis, Metabolism and Digestive Diseases. The procedure used was that described in the instructions, except that the antibody-hormone complex was precipitated with poly(ethylene glycol) as described by Desbuquois & Aurbach (1971). The somatotropin content of the samples was determined from the standard curve and expressed in terms of ng of NIAMDD-GH-RP-1 standard/ml of incubation medium.

Measurement of apparent changes in activities of phospholipase A and prostaglandin synthase

Anterior pituitary glands were removed from male rats and prelabelled by incubation for 1 h in 1 ml of Krebs-Ringer bicarbonate medium/11 mM-glucose

(Betteridge & Wallis, 1973), containing 10 μ Ci of [3 H]arachidonic acid/ml (135 Ci/mmol). The glands were then removed from the medium, bisected and the individual lobes were transferred to fresh Krebs-Ringer bicarbonate medium/11 mM-glucose, with or without various additions and modifications, and incubated at 37°C. Normally the K^+ concentration totalled 5.9 mequiv./litre. Where the effect of elevated K^+ concentration was being tested, this was raised to 59 mequiv./litre by the addition of KCl, and the NaCl concentration was lowered to obtain the same overall osmolarity. Where the $CaCl_2$ concentration was altered from 2.5 mM, the NaCl concentration was altered slightly for the same reason.

The redistribution of the [3 H]arachidonic acid during the second incubation was then measured. The tissue was transferred to 0.2 M-Tris/HCl, pH 7.4, at 0°C (0.25 ml/pituitary lobe), and homogenized rapidly before 4 vol. of chloroform/methanol (1:1, v/v) was added to precipitate protein and extract the lipid-soluble material. The organic phase was removed and dried *in vacuo*. The residue from this was resuspended in a small volume of chloroform/methanol (1:1, v/v.) for t.l.c. on Eastman Chromagen plates with benzene/dioxan/acetic acid (20:20:1, by vol.) as solvent and prostaglandins and arachidonic acid as markers. Phospholipids do not migrate from the origin in this system, but analysis of the material from the origin has been described elsewhere (Betteridge & Wallis, 1978) and it was shown that 95% of the radioactivity from this region is associated with phospholipids, especially phosphatidylethanolamine (approx. 50%). The areas corresponding to the standards of prostaglandins and arachidonic acid were cut out, immersed in scintillation fluid (Betteridge & Wallis, 1973) and counted for radioactivity. After subtracting the background radioactivity, the amount of radioactivity in each fraction was calculated. Although the prostaglandins E and F_a could be separated easily by this chromatographic system, for these studies the incorporation into both was combined. The results can be used to give an indirect indication of differences between the rates of phospholipase A action and prostaglandin synthase. All the tissue is preincubated together, so that we assume that the radioactivity is distributed similarly in the control and experimental tissue at the start of the second incubation. At the end of the second incubation, in the absence of added [3 H]arachidonic acid, the radioactivity already incorporated will be redistributed. Any differences between the distribution of radioactivity in the control and experimental samples will reflect different rates of redistribution and from these we can infer that the various enzyme activities involved have been altered.

Measurement of cell protein

The protein contents of the cells in each dish were measured by subjecting samples of the sonicated material to the method of Lowry *et al.* (1951) after dissolving it in 0.5M-NaOH. The prostaglandin E content of the cells was expressed as pmol of prostaglandin/mg of protein in each dish.

Results

Cyclic nucleotides and intracellular prostaglandin E concentration

In an attempt to relate changes in intracellular concentrations of cyclic AMP and cyclic GMP to prostaglandin concentration in anterior pituitary cells, dishes of these cells were treated with two agents known to increase cyclic nucleotide concentration. Isobutylmethylxanthine is known to inhibit cyclic nucleotide phosphodiesterase, and 8-bromo cyclic AMP has the effect of increasing cyclic AMP concentration either by inhibition of the phosphodiesterase or because it is hydrolysed to form cyclic AMP after it enters the cells. The methylxanthines, such as isobutylmethylxanthine and theophylline, have been shown (Schofield, 1967; Wilber *et al.*, 1968) to stimulate somatotropin release from anterior pituitary tissues. Similarly a wide variety of cyclic AMP derivatives have been shown (Hertelendy *et al.*, 1971; Lemay & Labrie, 1972) to stimulate the release of somatotropin and the other pituitary hormones.

Fig. 1 shows that both 8-bromo cyclic AMP and isobutylmethylxanthine stimulate prostaglandin E concentration in these cells over a short time course. The effect of 1mM-8-bromo cyclic AMP is short-lived, being only apparent after 10 min of incubation. The effect of 0.5mM-isobutylmethylxanthine is longer lasting. It causes elevated prostaglandin E concentrations (approx. 2-fold) for at least 20 min of incubation.

Effect of elevated K⁺ concentration on prostaglandin E accumulation

Elevated K⁺ concentrations are known to stimulate the release of most anterior pituitary hormones (Kraicer, 1975). The effect of increasing the K⁺ concentration by 10-fold was tested on dispersed pituitary cells in culture. It was confirmed that the release of both somatotropin and prolactin was increased under these conditions [$182 \pm 27\%$ and $145 \pm 16\%$ (means $\pm$ s.e.m.) of control respectively after 10 min (5 experiments)]. Fig. 2 shows that there is a rapid increase in prostaglandin E concentration within 5 min of the change of medium and they remained high for a further 25 min, returning to control values after about 45 min of incubation.

Effect of ionophore A23187 on intracellular prostaglandin E concentration and somatotropin release

Ionophores, such as A23187, increase membrane permeability to bivalent ions such as Ca²⁺. If the effect of elevated K⁺ concentration on prostaglandin production was a result of increased membrane permeability, then we might expect A23187 to stimulate intracellular prostaglandin E concentration also. Various concentrations of A23187 were added to anterior pituitary cells and the intracellular prostaglandin E concentrations were measured (Fig. 3a). After 5 min incubation, all the ionophore concentrations between 0.2 and 5 μ M caused significant increases in the prostaglandin E concentration, 2 μ M being the most effective.

Fig. 1. Effect of cyclic nucleotides on intracellular prostaglandin E concentrations

Dispersed anterior pituitary cells were incubated for various times with 1mM-8-bromo cyclic AMP (■) or 0.5mM-isobutylmethylxanthine (○) or neither (□). The amount of prostaglandin E in the cells was measured as described in the text. The results are means $\pm$ s.e.m. obtained from four separate experiments.

Fig. 2. Effect of elevated K^+ concentrations on prostaglandin E concentrations

Dispersed anterior pituitary cells were incubated for various times in media containing either 5.9 mM- (■) or 59 mM- (□) K^+ . The prostaglandin E concentration in the cells was measured as described in the text. The results shown are means $\pm$ S.E.M. from four separate experiments.

We have shown previously (Betteridge & Wallis, 1978, 1979) a good correlation between intracellular prostaglandin E concentration and somatotropin production under a variety of conditions. When the rate of somatotropin release was measured in the same experiments with A23187 described above, the results showed there was also an increased release of somatotropin (Fig. 3b). All the A23187 concentrations tested (from 0.2 to 10 μ M) significantly ($P < 0.05$) increased the amount of somatotropin released. Again the most effective concentration was 2 μ M. By contrast with the effect of prostaglandin E concentrations, there was no significant decrease of the effect on somatotropin release with 5 or 10 μ M-A23187.

Changes in [3H]arachidonic acid distribution related to the ionic composition of the medium

Since we have shown previously (Betteridge & Wallis, 1978) that changes in precursor availability affect the rate of prostaglandin production, we investigated whether the increase in prostaglandin

Fig. 3. Effect of A23187 on prostaglandin E concentration and somatotropin release

Dispersed anterior pituitary cells were incubated for 5 min with various concentrations of A23187. The intracellular prostaglandin E concentrations (a) and the amounts of somatotropin released into the medium (b) were measured as described in the text. The results are means $\pm$ S.E.M. obtained from four separate experiments.

concentration in the presence of elevated K^+ concentration was a result of increased phospholipase A activity. The redistribution of [3H]arachidonic acid, during a 10 min incubation, was measured in anterior pituitary glands prelabelled for 1 h. After the second incubation, there was a significantly ($P < 0.01$) lower amount of radioactivity associated with the phospholipid fraction from the tissue exposed to elevated K^+ concentration, compared with control (Table 1). Similarly the amounts of free [3H]arachidonic acid and radioactively labelled prostaglandins were sig-

Table 1. *Effect of various changes in the incubation medium on the distribution of [³H]arachidonic acid*
 Rat hemipituitaries were incubated for 1 h in Krebs–Ringer bicarbonate/11 mM-glucose, containing 10 μ Ci of [³H]arachidonic acid (135 Ci/mmol)/ml. The medium was changed to fresh Krebs–Ringer bicarbonate/glucose with or without the various modifications or additions indicated and the tissue was incubated for a further 10 min. The amount of radioactivity in various lipid fractions was determined as described in the text. The results are means $\pm$ S.E.M. for three experiments. * $P < 0.05$; ** $P < 0.1$, compared with the values obtained using 2.5 mM-Ca²⁺ and 5.9 mM-K⁺.

Ca ²⁺ (mM)	K ⁺ (mM)	Additions	Radioactivity (d.p.m.) in		
			Phospholipids	Prostaglandins E + F	Arachidonic acid
2.5	5.9	None	5500 $\pm$ 160	1750 $\pm$ 150	1010 $\pm$ 130
2.5	5.9	None	3780 $\pm$ 490*	2480 $\pm$ 190*	1669 $\pm$ 170*
—	5.9	EDTA (1 mM)	7300 $\pm$ 190**	1200 $\pm$ 170*	430 $\pm$ 90*
10	5.9	EDTA (1 mM)	5920 $\pm$ 370	1740 $\pm$ 240	940 $\pm$ 140
2.5	5.9	Isobutylmethylxanthine (0.5 mM)	5246 $\pm$ 230	2090 $\pm$ 110	1050 $\pm$ 100

nificantly higher ($P < 0.01$). This can be interpreted to mean that more of the [³H]arachidonic acid was being released from phospholipid in the experimental tissue and this would imply that there was increased phospholipase A activity in the presence of elevated K⁺ concentration.

If CaCl₂ is omitted from the incubation medium for glands pre-labelled with [³H]arachidonic acid and EDTA is added, there are significant changes in the distribution of [³H]arachidonic acid (Table 1). There is a significantly ($P < 0.01$) higher amount of radioactivity associated with the phospholipid fraction (33% higher), whereas the radioactivity incorporated into prostaglandins and that remaining as free arachidonic acid is lowered (by 33 and 57% respectively). If, on the other hand, 10 mM-CaCl₂ is added in addition to EDTA, the distribution of radioactivity is not significantly different from that measured in the presence of 2.5 mM-CaCl₂ (Table 1). Furthermore, the addition of isobutylmethylxanthine had no detectable effect on the phospholipase A activity, since the proportion of the radioactivity found associated with phospholipids was unchanged (Table 1). There was a small (19%) but statistically non-significant increase in the amount of radioactivity in the prostaglandins, compared with free arachidonic acid, which may reflect the increased prostaglandin concentrations shown in Fig. 1.

Discussion

The results show that two substances (8-bromo cyclic AMP and isobutylmethylxanthine) that are both known to increase intracellular cyclic AMP concentrations in many tissues (probably by inhibition of cyclic nucleotide phosphodiesterase) are capable of increasing the concentration of prosta-

glandin E in anterior pituitary glands. The evidence suggests that this is an effect on prostaglandin synthesis directly, or possibly on prostaglandin degradation, since no changes in phospholipase A activity were detected with the indirect assay described here. At this stage it is not possible to say whether cyclic AMP itself interacts with the prostaglandin synthase, or acts via some intermediary. An investigation using purified prostaglandin synthase may answer this question.

Elevation of extracellular K⁺ concentrations causes increased secretion of probably all the anterior pituitary hormones (McCann & Porter, 1969), and this is thought to occur at least partly as a result of depolarization of the plasma membrane (Douglas & Poisner, 1964). The results show that elevated K⁺ concentrations cause a rapid rise in intracellular prostaglandin E concentrations. Furthermore, this appears to be associated with increased phospholipase A activity, since an increased transfer of radioactivity from phospholipids to prostaglandins and free arachidonic acid was measured in the presence of elevated K⁺ concentrations.

It has been suggested that Ca²⁺ plays a part in a number of secretion processes (Rubin, 1970). The results show that incubation of the tissue in Ca²⁺-free medium, with the addition of EDTA, results in less [³H]arachidonic acid being redistributed suggesting that phospholipase A activity was lower, compared with control incubation. From this it is inferred that there is a requirement for bivalent ions such as Ca²⁺ for the appropriate phospholipase A activity. However, replacing the CaCl₂ to a concentration of 10 mM overcame the effect of EDTA. From this, one can conclude that Ca²⁺ is probably the ion required for phospholipase A activity. The stimulation of intracellular prosta-

glandin E concentrations by the ionophore A23187 may also result from the increased entry of bivalent ions such as Ca^{2+} into the cells. It is possible to combine these various observations in a single explanation. One major effect of both A23187 and elevated extracellular K^{+} concentration is to increase membrane permeability to bivalent ions. If this then results in an increased influx of Ca^{2+} ions, for example, this could stimulate phospholipase A activity, as we have shown that there is a requirement for Ca^{2+} ions for optimal activity. Increased phospholipase A activity then results in increased prostaglandin precursor availability and hence increased prostaglandin production.

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References

- Ashby, J. P. & Speake, R. N. (1975) *Biochem. J.* **150**, 89–96
- Barden, N. & Betteridge, A. (1977) *J. Endocrinol.* **75**, 277–283
- Betteridge, A. & Wallis, M. (1973) *Biochem. J.* **134**, 1103–1113
- Betteridge, A. & Wallis, M. (1977) *Biochem. Soc. Trans.* **5**, 224–226
- Betteridge, A. & Wallis, M. (1978) *Biochem. J.* **176**, 319–323
- Betteridge, A. & Wallis, M. (1979) *J. Endocrinol.* **80**, 239–248
- Bicknell, R. J. & Schofield, J. G. (1976) *FEBS Lett.* **68**, 23–26
- Desbuquois, B. & Aurbach, G. D. (1971) *J. Clin. Endocrinol. Metab.* **33**, 732–738
- Douglas, W. W. & Poisner, A. M. (1964) *J. Physiol. (London)* **172**, 1–18
- Foreman, J. C., Mongor, J. L. & Gomperts, B. D. (1973) *Nature (London)* **245**, 249–251
- Gautvik, K. M. & Tashjian, A. H. (1973) *Endocrinology* **93**, 793–799
- Hertelendy, F., Todd, H., Peake, G. T., Machlin, L. J., Johnson, G. & Pounds, G. (1971) *Endocrinology* **89**, 1256–1262
- Kraicer, J. (1975) in *The Anterior Pituitary* (Tixien Vidal, A. & Farquhar, M., eds.), pp. 21–43, Academic Press, New York
- Labella, F., Dular, R., Vivian, S. & Queen, G. (1973) *Biochem. Biophys. Res. Commun.* **52**, 786–791
- Labrie, F., Pelletier, G., Borgeat, P., Drouin, J., Ferland, L. & Bélanger, A. (1976) in *Frontiers in Neuroendocrinology* (Martini, L. & Ganong, eds.), vol. 4 pp. 63–93, Raven Press, New York
- Lemay, A. & Labrie, F. (1972) *FEBS Lett.* **20**, 7–10
- Lowry, O., Rosebrough, N., Farr, A. & Randall, R. (1951) *J. Biol. Chem.* **193**, 265–275
- McCann, S. M. & Porter, J. C. (1969) *Physiol. Rev.* **49**, 240–284
- Rubin, R. D. (1970) *Pharmacol. Rev.* **22**, 389–428
- Scarpa, A. & Inesi, G. (1972) *FEBS Lett.* **22**, 273–276
- Schofield, J. G. (1967) *Nature (London)* **215**, 1382–1383
- Vale, W., Grant, G., Amoss, M., Blackwell, R. & Guillemin, R. (1972) *Endocrinology* **91**, 562–572
- Wilber, J., Peake, G., Mariz, J., Utiger, R. & Daughaday, W. (1968) *Clin. Res.* **16**, 277