Changes in cyclic AMP and cyclic GMP concentrations during the action of 5-hydroxytryptamine on an insect salivary gland

John P. HESLOP and Michael J. BERRIDGE

A.R.C. Unit of Invertebrate Chemistry and Physiology, Department of Zoology, Downing Street,
Cambridge CB2 3EJ, U.K.

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Salivary glands from adult blowflies (*Calliphora erythrocephala* Meigen) were studied *in vitro*. The time course of changes in cyclic AMP content of the glands was followed at different concentrations of 5-hydroxytryptamine. There was an immediate biphasic rise and fall in cyclic AMP content, followed by a slower rise and subsequent gradual decline. The initial rise preceded the onset of fluid secretion by the glands. Rises in cyclic AMP content were inhibited by compound RMI 12330 A (an adenylate cyclase inhibitor) and were halted after about 15–20 s if the glands were deprived of Ca^{2+} . Theophylline (a phosphodiesterase inhibitor) abolished the decline phase of the fast response. Losses of cyclic AMP from the glands either to the bathing medium or to the saliva were small and could not account for the rapid fall found. Evidence is presented that cyclic GMP is not involved in the process of initiating secretion in the blowfly salivary gland.

The complex sequence of events that make up stimulus–secretion coupling is now beginning to be understood (Berridge, 1975, 1980). In particular, simultaneous (and probably interacting) changes in the intracellular concentrations of Ca^{2+} and of cyclic nucleotides have been described after stimulation of many cell types by hormones and neurotransmitters (Berridge, 1980). The blowfly salivary gland provides a convenient model for the study of these phenomena in a serotonin-sensitive system. The inflow of Ca^{2+} that immediately follows challenge of the glands by 5-hydroxytryptamine *in vitro* has been extensively studied and there is evidence that cyclic AMP, too, may be involved in the events associated with salivary secretion (Prince *et al.*, 1972). No evidence was available as to whether cyclic GMP might be implicated and it was necessary to answer this question and to investigate in greater detail the role of cyclic AMP by using the more sensitive radioimmunoassay techniques that are now available.

Methods

Blowflies (*Calliphora erythrocephala* Meigen) were reared in the laboratory. Flies of both sexes were used. That part of the salivary gland that lies in the abdomen was taken out for analysis and stored in 5 cm plastic Petri dishes containing 10 ml of saline solution. The same saline was used for dissection,

storage and all experiments on the glands except where stated. It contained 128 mM- Na^+ , 10 mM- K^+ , 2 mM- Ca^{2+} , 2 mM- Mg^{2+} , 3.8 mM-phosphate, 2.7 mM-glutamate, 2.8 mM-dl-malate, 10 mM-Tris, 10 mM-glucose and a trace of Phenol Red. The pH of the solution was adjusted to 7.2. Normally six dishes, each containing six glands, were prepared at a time. They were protected from direct sunlight, but kept at room temperature (16–24°C throughout). No dish contained two glands from the same animal. During experiments all six glands were grasped together with stainless-steel forceps, immersed in drug solutions or saline for the appropriate time and then transferred to the pestle of a 0.1 ml glass homogenizer (Jencons Scientific, Hemel Hempstead, Herts., U.K.). The homogenizer tube contained 0.1 ml of 0.4 M- HClO_4 and was kept in ice. The pestle was plunged rapidly into the HClO_4 and the glands were homogenized by hand. The homogenate was neutralized with KOH, kept in ice for at least 30 min, and the tube was centrifuged at 2000 g for 15 min at 4°C. The supernatant was adjusted to a final concentration of 0.05 M-sodium acetate, pH 6.5, ready for analysis. This single-extraction procedure recovered 89% of the cyclic nucleotides in the tissue. Techniques for measurements of the rate of fluid secretion and the collection of samples of saliva and bathing medium were as described by Berridge & Prince (1971).

Cyclic GMP was assayed by using kits based on the radioimmunoassay method of Steiner *et al.* (1972) with acetylation according to the manufacturer's instructions (NEN Chemicals G.m.b.H., D-6072, Dreieich, Germany). The whole extract from six glands was used in a single assay tube. Assays for cyclic AMP were carried out on suitably diluted portions of the extract from six glands using NEN kits (as for cyclic GMP) in a few cases, or by a slight modification of the Steiner *et al.* (1972) procedure with antiserum kindly supplied by Dr. A. L. Steiner (after the incubation 0.7 mg of γ -globulin was added and the precipitation done with alcohol to a final concentration of 85%). In all cases the precipitated protein plus antiserum was dissolved in 50 μ l of Soluene (Packard Instrument, Caversham, Berks., U.K.), and dispersed in 3.5 ml of Bray's solution for liquid-scintillation counting. Compound RMI 12330 A was supplied by Merrell-National Laboratories, Cincinnati, OH 45215, U.S.A.

Results

After application of 0.1 μ M-5-hydroxytryptamine to an isolated salivary gland, there followed a lag period of about 25 s, after which the rate of secretion of saliva increased quickly, reaching a maximum by about 60 s (Fig. 1). Glands continued to secrete at the maximum rate for long periods provided the stimulus was maintained. Particular attention was paid to cyclic nucleotide concentrations in the glands during the first minutes after stimulation.

Cyclic GMP determinations

The resting content of cyclic GMP in blowfly salivary glands is low. It was 2.1 ± 0.17 fmol/gland $\pm$ S.E.M. ($n = 13$) based on 77 groups of six glands from 13 different cultures of flies. A surprising finding was the variation between cultures. The extreme values of cyclic GMP were 1.3 ± 0.18 (6) and 3.6 ± 0.11 (6) fmol/gland based on six groups each of six glands. The effects of experimental treatments on cyclic GMP content have therefore been expressed as proportions of the appropriate controls.

When salivary glands were stimulated with

0.1 μ M-5-hydroxytryptamine (a dose five times that which would induce maximal fluid secretion), there was a small but significant increase in the cyclic GMP concentration (Table 1). To explore this further the experiment was repeated at different dosages and also in the presence of two inhibitors of cyclic nucleotide phosphodiesterase. Although statistically significant changes in cyclic GMP content were observed (Table 2), they were small even in the presence of the inhibitors, and there was no evidence for a greater increase in cyclic GMP when the dosage of 5-hydroxytryptamine was raised.

An alternative approach was to stimulate the synthesis of cyclic GMP by direct action on guanylate cyclase. Very rapid increases in cyclic GMP in intact cells have been found after exposure to ascorbate (Schoepflin *et al.*, 1977; Laychock & Hardman, 1978), nitrite (Katsuki & Murad, 1977), nitroprusside (Laychock & Hardman, 1978) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (DeRubertis & Craven, 1977). These agents were applied to salivary glands at the concentrations that have produced maximal effects in other systems and the

Fig. 1. Short-term response of salivary glands to treatment with 5-hydroxytryptamine

Glands were stimulated with 0.1 μ M-5-hydroxytryptamine at time zero and the rate of secretion (●) or content of cyclic AMP (O) were determined at different times.

Table 1. Time course of the effect of 0.1 μ M-5-hydroxytryptamine on relative concentrations of cyclic GMP in blowfly salivary gland

Time after challenge with 5-hydroxytryptamine (s)	Cyclic GMP experimental/control concentration ratio	P (t test)
18	0.91	—
40	0.89	—
60	1.46	<0.01
120	1.27	<0.02

results are shown in Table 3. A slight increase in cyclic GMP concentration was observed after treatment with ascorbic acid, but since the analytical method could only allow detection of a change of +20% for six replicates each of control and experimental, this observation is of doubtful significance. With the other agents no effect at all was detectable.

Because the glands might have acetylcholine receptors, the effect of 10 μM-carbachol was also investigated. The amount of cyclic GMP in the glands 1 min after treatment with 10 μM-carbachol was 0.87 times that of the controls (no significant difference).

There remained the possibility that cyclic GMP was being synthesized in the glands at an enhanced rate after stimulation, but was being lost from the cells either to the bathing medium or in the outflowing saliva. To test this, six groups of six glands were dipped in saline containing 0.1 μM-5-hydroxytryptamine and 1 mM-theophylline. They were at once transferred to the pestle of a homogenizer in a drop of the drug-containing saline and incubation was allowed to proceed in the hanging drop for a total of 1 min at 26°C before homogenization as before. The extract therefore contained both the glands and any cyclic nucleotide that might have been exported from them. The ratio of experimental to control cyclic GMP content was 1.95 ($P < 0.02$ by t test), which agrees closely with the analogous experiment on glands alone (Table 2).

We concluded that losses of cyclic GMP from the glands could not account for the failure to find substantial increases in the amount of this nucleotide after stimulation by 5-hydroxytryptamine (Tables 1–3).

Cyclic AMP determinations

In comparison with cyclic GMP there is a high concentration of cyclic AMP in blowfly salivary glands [79.5 ± 4.96 fmol/gland $\pm$ s.e.m. ($n = 39$), based on 39 groups usually of six glands]. On exposure of the glands to 5-hydroxytryptamine there were substantial and rapid changes in cyclic AMP (Fig. 1). The initial rise in cyclic AMP (Fig. 1) clearly preceded the onset of fluid secretion, which as noted above was always delayed by at least 25 s. When the dosage of 5-hydroxytryptamine was 1 nM no secretion of saliva was visible until 40 s after treatment. It was noted that the initial increase in cyclic AMP ceased after about 25 s and that it did so at this time regardless of whether a very high or relatively low intracellular concentration of the nucleotide had been reached. A dose-response curve was prepared with, as the criterion of response, the slope of the initial rapid rise in cyclic AMP between 10 and 20 s after exposure to 5-hydroxytryptamine (Fig. 2). The threshold of the cyclic AMP response corresponded closely to the minimum dosage required for fluid secretion (Fig. 9). As has been found in other systems, a maximum physiological response can be obtained at dosages of the drug that

Table 2. The effect of 1 min exposures to 5-hydroxytryptamine on cyclic GMP concentrations in blowfly salivary glands
*, Difference from control significant at $P < 0.01$ by t test. Abbreviation used: IBMX, isobutylmethylxanthine.
Three experiments were done at 0.1 μM-5-hydroxytryptamine, one of which did not show a significant change.

[5-Hydroxytryptamine] (μM)	Cyclic GMP experimental/control concentration ratio		
	Normal saline	1 mM-Theophylline	1 mM-IBMX
0	1.0	0.91	0.79
0.03	1.19	—	—
0.10	1.27*, 1.46*, 0.90	2.15*	1.49*
0.32	1.06	—	—
1.0	0.85	2.12*	—
3.2	1.31	—	—

Table 3. The effect of various agents at 1 mM concentration on the concentration of cyclic GMP in blowfly salivary gland

Drug	Time (min)	Cyclic GMP experimental/ control concentration ratio	Significance (t test)
Ascorbic acid	1	1.34	$P < 0.02$
Nitrite	1	0.93	—
Nitroprusside	1	1.01	—
	2	1.02	—
N-Methyl-N'-nitro-N-nitrosoguanidine	1	1.08	—

Fig. 2. Dose-related response of salivary glands to 5-hydroxytryptamine

The slope of the time-response curve between 10 and 20 s after stimulation by 5-hydroxytryptamine in normal saline (■) and the rate of secretion of saliva from 1 to 4 min after stimulation (●) are shown.

Fig. 3. Inhibition of cyclic AMP accumulation in salivary glands by compound RMI 12330 A

Curve (a) shows the rate of accumulation of cyclic AMP in normal glands after stimulation with $0.1 \mu\text{M}$ -5-hydroxytryptamine. The experiment was repeated in the presence of 0.1 mM - (curve b) or 0.5 mM -compound RMI 12330 A (curve c).

give only a modest increase in cyclic AMP. At higher dosages the rate of accumulation of cyclic AMP was decreased, but not enough to affect fluid secretion.

An increase in intracellular cyclic AMP might be due either to increased synthesis or to decreased catabolism. Compound RMI 12330 A was developed as an inhibitor of adenylate cyclase by Merrell-National Laboratories, and has been reported to have effects consistent with this action by Siegel & Wiech (1976) and Ilundain & Naftalin (1979). In our studies at a concentration of 0.1 mM it partly inhibited the initial increase in cyclic AMP that follows stimulation by 5-hydroxytryptamine and at 0.5 mM the increase was abolished (Fig. 3). There were also effects on fluid secretion (Fig. 4). At $30 \mu\text{M}$ the inhibition of secretion was slight, but a substantial, partially reversible inhibition of the flow of saliva was found when the dosage was increased to 0.1 mM . Even the complete inhibition caused by 0.5 mM -compound RMI 12330 A was reversible to a limited extent. The latter dose of compound RMI 12330 A had little effect on the resting-gland concentration of cyclic AMP, which was decreased only to about one-half the normal value after 30 min of continuous exposure to the drug (Table 4). This finding suggests that the activity of phosphodiesterase in the resting glands must also be low, a point that has been further investigated by using theophylline as inhibitor.

The marked decrease in cyclic AMP concentration that occurs between 30 and 120 s after stimulation with 5-hydroxytryptamine (Fig. 1) represents a substantial loss or destruction of the nucleotide.

Fig. 4. Effects of compound RMI 12330 A on 5-hydroxytryptamine-induced salivary secretion

Control salivary glands were treated *in vitro* with $0.1 \mu\text{M}$ -5-hydroxytryptamine and the rate of secretion of saliva was determined (O). The experiment was repeated in the presence of $30 \mu\text{M}$ - (▼), 0.1 mM - (Δ) and 0.5 mM - (●) compound RMI 12330 A. In two cases partial reversal of the inhibiting effects of compound RMI 12330 A were obtained when this drug was removed by washing with $0.1 \mu\text{M}$ -5-hydroxytryptamine plus saline at the times indicated by the arrows.

To study the possible egress of cyclic AMP, glands were stimulated with $0.1 \mu\text{M}$ -5-hydroxytryptamine under liquid paraffin. The bathing medium and saliva were collected every 3 min and analysed for cyclic AMP. There was a slight egress

Table 4. Effects of compound RMI 12330 A on the amount of cyclic AMP in salivary glands after 30 min incubations with or without 0.1 μM-5-hydroxytryptamine

Results shown are means for triplicate determinations expressed as proportions of the relevant control.

5-Hydroxytryptamine concn.	Cyclic AMP (% of control)			
	[Compound RMI 12330 A] (μM)	...	0 (control)	30 500
0			100	72 47
0.1 μM			624	177 45

of cyclic AMP to the bathing medium (0.8 fmol/gland per min), which increased to 3.0 ± 0.67 fmol/gland per min ($n = 4$) after stimulation. Similar experiments carried out with 0.1 μM-5-hydroxytryptamine plus 10 mM-theophylline (both to increase the intracellular cyclic AMP concentration and to inhibit any cyclic nucleotide phosphodiesterase that might contaminate saliva collected from cut glands) showed egress of 7.9 ± 1.28 fmol/gland per min ($n = 8$). The actual decrease in cyclic AMP concentration in the glands during the decline phase of the biphasic response between 30 and 120 s after stimulation was 490–925 fmol (Fig. 1). Clearly it was necessary to look elsewhere for the immediate cause of the decline phase of the initial cyclic AMP response.

Cyclic nucleotide phosphodiesterase in glands was inhibited by a 30 s preincubation in 1 or 10 mM-theophylline and 0.1 μM-5-hydroxytryptamine plus theophylline was then applied. All drugs were dissolved in normal saline that contains 2 mM- Ca^{2+} . It was found that the fast-decline phase of the cyclic AMP response was delayed and slowed by 1 mM-theophylline (Fig. 5) and abolished when theophylline was raised to 10 mM (Fig. 6). The slope of the fast-increasing phase of the cyclic AMP response was not increased when theophylline was present before and during stimulation as compared with 5-hydroxytryptamine alone. It was also found that theophylline (10 mM for 6 min) alone had little effect on the concentration of cyclic AMP in resting glands (Fig. 6). These results suggest that inhibition of phosphodiesterase action could not be a significant factor in bringing about the initial rise in cyclic AMP concentration.

An immediate response to stimulation of blowfly salivary glands by 5-hydroxytryptamine is a rapid influx of Ca^{2+} into the cells (Berridge & Lipke, 1979; Fain & Berridge, 1979a,b). To prevent this and to deplete intracellular reserves of Ca^{2+} , glands were dissected and stored in Ca^{2+} -free saline containing 100 μM-EGTA. Prolonged treatments (up to 3 h) with this solution did not affect the concentration of cyclic AMP in resting glands. When glands kept for 1–2 h in Ca^{2+} -free saline plus EGTA were exposed to 0.1 μM-5-hydroxytryptamine there was an immediate rapid rise in cyclic AMP that continued

Fig. 5. Effect of 5-hydroxytryptamine and 1 mM-theophylline on cyclic AMP concentrations in the presence or absence of Ca^{2+}

Glands were pretreated with 1 mM-theophylline (open bar) for 30 s before the addition of 0.1 μM-5-hydroxytryptamine (solid bar) either in the presence of 2 mM- Ca^{2+} (●) or in a Ca^{2+} -free medium containing 100 μM-EGTA (Δ). Control glands (▼) were treated with theophylline alone. In separate experiments glands treated with 1 mM- or 10 mM-theophylline in a Ca^{2+} -free medium (100 μM-EGTA) showed no detectable change in cyclic AMP content. The broken line shows the effect of 5-hydroxytryptamine without theophylline (from Fig. 1).

Fig. 6. Effect of 5-hydroxytryptamine and 10 mM-theophylline on cyclic AMP concentrations in the presence or absence of Ca^{2+}

Glands were treated in the same way as described in Fig. 5, except that the concentration of theophylline was increased to 10 mM. The broken line shows the effect of 5-hydroxytryptamine without theophylline (from Fig. 1).

for 20s and was followed by partial reversal (Fig. 7). The pattern of response, however, differed from that found in the presence of Ca^{2+} . The reversal of change in cyclic AMP concentrations occurred earlier and was more abrupt under Ca^{2+} -free conditions. When the cyclic AMP concentration in the glands reached about 200 fmol/gland it stabilized for a long period and the second, slower, increase did not appear. At 40 and 77 min after the onset of 5-hydroxytryptamine treatment we found 168 and 138 fmol of cyclic AMP/gland respectively.

The egress of cyclic AMP from the glands under Ca^{2+} -free conditions was followed in 12 glands, singly exposed to $0.1 \mu\text{M}$ -5-hydroxytryptamine. Over a 15 min period saliva production decreased from 23.4 nl/min (average of first 5 min) through 18.2 to 12.2 nl/min (average of last 5 min). The average amount of cyclic AMP in the saliva was 14.2 ± 2.29 fmol/gland per 15 min ($n = 12$), which, with an average saliva volume of 19.4 ± 3.58 nl/gland per min $\pm$ s.e.m., corresponds to a concentration of $0.05 \mu\text{M}$. Egress to the bathing medium was 37.5 ± 3.58 fmol/gland per 15 min ($n = 12$), but most of the cyclic AMP [248 ± 60.6 fmol/gland ($n = 12$)] remained within the glands at the end of the incubation period. This represents an intracellular concentration of 3–7 μM -cyclic AMP.

Prolonged treatment with $100 \mu\text{M}$ -EGTA in the absence of Ca^{2+} might have seriously affected the ability of the glands to respond to 5-hydroxytryptamine at all. As a test of this possibility such glands were exposed to $0.1 \mu\text{M}$ -5-hydroxytryptamine for 10 min and then transferred to the same concentration of the hormone in normal saline

Fig. 7. The influence of Ca^{2+} on the response of salivary glands to 5-hydroxytryptamine

Glands were dissected and stored for 1–2 h at room temperature in Ca^{2+} -free saline containing 0.1 mM-EGTA. Groups of six glands were extracted after different periods in $0.1 \mu\text{M}$ -5-hydroxytryptamine in Ca^{2+} -free saline (Δ). In some cases glands after 10 min stimulation in Ca^{2+} -free solution were transferred to $0.1 \mu\text{M}$ -5-hydroxytryptamine in normal saline containing 2 mM- Ca^{2+} ($\bullet$).

(no EGTA, 2 mM- CaCl_2). A second typical biphasic response followed by a slower rise in cyclic AMP concentration was found, albeit of decreased amplitude, indicating that the cyclic AMP-generating system had not been destroyed by the pretreatment (Fig. 7). It follows that the failure to find the second rise in cyclic AMP concentration that might have been expected between 2 and 20 min after challenge with 5-hydroxytryptamine was due to lack of Ca^{2+} .

The effect of removing Ca^{2+} on cyclic AMP formation was also tested during treatment with theophylline (Figs. 5 and 6). At both theophylline concentrations the rise in cyclic AMP during stimulation with $0.1 \mu\text{M}$ -5-hydroxytryptamine was severely curtailed. Attention was paid to the rise in cyclic AMP concentration that occurs between 2 and 20 min after stimulation (Fig. 8). This slower rise depended on the presence of Ca^{2+} and was also inhibited by compound RMI 12330 A (Table 4).

It has been reported that prolonged and excessive stimulation with 5-hydroxytryptamine can bring about inactivation of the Ca^{2+} -gating response of blowfly salivary glands to the amine and that this inactivation is associated with the loss of phosphatidylinositol (Berridge & Fain, 1979). Furthermore, inactivation of this sort is reversible only if inositol is added to the incubation medium after removal of the

Fig. 8. Effects of prolonged and excessive stimulation by 5-hydroxytryptamine on cyclic AMP in salivary glands

Glands were exposed at room temperature in the dark to $10 \mu\text{M}$ -hydroxytryptamine for 2 h (indicated by the horizontal bar), washed with normal saline for 30 min and again challenged with $10 \mu\text{M}$ -5-hydroxytryptamine (indicated by the horizontal bar). Groups of six glands were taken for analysis at different times. Error bars indicate $\pm$ s.e.m. (n).

stimulus (Berridge & Fain, 1979; Fain & Berridge, 1979b). A similar experiment was undertaken to study the cyclic AMP response to 5-hydroxytryptamine. Glands were placed in $10\mu\text{M}$ -5-hydroxytryptamine for 2 h and then washed with normal saline. After a 30 min recovery period they were again transferred to $10\mu\text{M}$ -5-hydroxytryptamine for up to 30 min. The high concentration of 5-hydroxytryptamine ($10\mu\text{M}$) produced an initial cyclic AMP response (Fig. 8) very similar to that found with $0.1\mu\text{M}$ (Fig. 1). After the biphasic fast response, there was a slower rise to a maximum at 20 min. The actual concentration reached was variable, as shown by the $\pm$ s.e.m. bar in Fig. 8 at 30 min. In individual glands treated with $0.1\mu\text{M}$ -5-hydroxytryptamine for 30 min it was 453 ± 60 fmol/gland $\pm$ s.e.m. ($n = 10$) so the variation is not just a characteristic of gross overstimulation. The decline in cyclic AMP concentration between 30 min and 120 min, however, was significant ($P < 0.001$) and a similar effect was also found at $0.1\mu\text{M}$ -5-hydroxytryptamine. On washing there was a very rapid decrease in cyclic AMP concentration, reaching resting-gland values within 30 s. Recovery periods shorter than 30 s were

not studied. A second challenge with $10\mu\text{M}$ -5-hydroxytryptamine after 30 min recovery produced a renewed biphasic response, showing that the mechanisms controlling cyclic AMP concentrations had not been inactivated by the lengthy pre-treatment. Two other experiments were done on glands that had been treated with $10\mu\text{M}$ -5-hydroxytryptamine for 2 h and allowed to recover for 30 min. First a dose-response curve of fluid secretion was prepared (Fig. 9). This showed a normal threshold of response, but a decreased fluid secretion, in the fatigued glands. Secondly, the dose necessary to produce a doubling of cyclic AMP concentration relative to controls at 20 or 30 min after application of 5-hydroxytryptamine was determined. It was $10\mu\text{M}$ in normal glands, but $30\mu\text{M}$ in the fatigued glands, suggesting that the threshold of stimulation of adenylate cyclase activity might have been increased by prolonged exposure to the hormone.

Discussion

Cyclic GMP

In our view the small increases in intracellular cyclic GMP concentration that occur after treatment of the glands with 5-hydroxytryptamine do not provide sufficient evidence to postulate an involvement of cyclic GMP in stimulus-secretion coupling. They would, for example, be plausibly explained if the insect adenylate cyclase had a slight cross-reactivity towards guanosine triphosphate. This would also explain why no extra cyclic GMP accumulated on stimulation in the presence of theophylline (we could say that the available substrate controlled the concentration of cyclic GMP attainable).

Stimulation of blowfly salivary glands by 5-hydroxytryptamine produces within 30 s a copious flow of saliva, but this secretory response is preceded by two distinct intracellular phenomena. There is an early and rapid inflow of Ca^{2+} via the basal membrane (Berridge & Lipke, 1979), and as was shown by Prince *et al.* (1972) and in the present paper there are effects on the concentration of cyclic AMP in the glands. We have found that 5-hydroxytryptamine induces a swift but transient rise in cyclic AMP in the glands, which is followed by a fall and a slower rise. Early effects on cyclic AMP, which precede the physiological response, have been found in many systems such as fat-cells (Manganiello *et al.*, 1971; Burns *et al.*, 1972; Lang & Schwyzer, 1972), thyroid (Williams, 1972), adrenal cortex (Grahame-Smith *et al.*, 1967) and liver (Exton *et al.*, 1971). In addition to this temporal evidence, there is further support for the suggestion of a causal relationship between the rise in cyclic AMP concentration and the subsequent physiological response. The threshold concentration of 5-hydroxy-

Fig. 9. Fluid secretion by salivary glands at different concentrations of 5-hydroxytryptamine

Control glands (●) are compared with glands previously exposed to $10\mu\text{M}$ -5-hydroxytryptamine for 2 h and allowed to rest for 30 min in normal saline before testing (○).

tryptamine needed to induce secretion is also the same as that to bring about the cyclic AMP response. In the blowfly salivary gland, treatment with compound RMI 12330 A (a putative inhibitor of adenylate cyclase) not only prevents fluid secretion but also abolishes both the fast and the slow phases of cyclic AMP production.

These results thus provide further evidence for a role of cyclic AMP in the regulation of fluid secretion. Additional direct evidence has been obtained by studying 5-hydroxytryptamine analogues with which it is possible to distinguish between receptor effects on either cyclic AMP or Ca^{2+} (M. J. Berridge & J. P. Heslop, unpublished work). An increase in the intracellular concentration of cyclic AMP seems to be a prerequisite for any increase in fluid secretion.

The kinetics of the rise in cyclic AMP concentration in response to 5-hydroxytryptamine includes a rapid biphasic change that precedes a slower more steady increase. Early transients in cyclic AMP after stimulation are probably an expression of the feedback-control networks responsible for regulating the intracellular concentrations of second messengers. An obvious possibility concerns the role of Ca^{2+} in regulating cyclic nucleotide metabolism (Berridge, 1975; Wolff & Bronstrom, 1979). Biochemical studies have revealed that Ca^{2+} , acting through its intracellular receptor protein calmodulin, can activate both adenylate cyclase and phosphodiesterase. Considerable caution must be exercised in extrapolating such biochemical observations to the situation *in vivo* because the activity of these enzymes may also be sensitive to univalent cations such as K^+ (Davis & Daly, 1978). Another difficulty concerns the problem of trying to predict the intracellular concentration of Ca^{2+} during the different phases of stimulation. At this stage, therefore, our ability to interpret the precise biochemical basis of this biphasic response is somewhat limited.

The initial rise in cyclic AMP concentration is apparently independent of external Ca^{2+} . However, if there is no Ca^{2+} in the bathing medium, this initial rise is cut short. It is likely, therefore, that this temporary response in Ca^{2+} -free media may depend on a mobilization of internal Ca^{2+} . Electrophysiological studies on salivary glands have revealed that a normal response can be initiated by stimulation with 5-hydroxytryptamine in a Ca^{2+} -free medium, but is also quickly cut short. After a resting period, however, subsequent stimulation induces a renewed but truncated response and the sequence of stimulation and rest can be repeated many times (Berridge & Prince, 1975). The activation of adenylate cyclase in this salivary gland seems to require the continued presence of Ca^{2+} . The sharp cessation of cyclic AMP synthesis after less than 20 s of stimulation in

Ca^{2+} -free medium may reflect exhaustion of the available Ca^{2+} . Some confirmation for this view is derived from the observation that a typical biphasic response was induced when Ca^{2+} was re-admitted to glands that had been continuously stimulated in Ca^{2+} -free medium (Fig. 7).

The decline phase of the initial fast biphasic change in cyclic AMP concentration resembles that found in other systems (Perkins *et al.*, 1978; Lefkowitz & Williams, 1978). Such transient effects have been attributed to desensitization of the receptor-response system, but this seems unlikely to be the case in this salivary gland because immediately after this biphasic response there was a second rise in cyclic AMP concentration (Figs. 1 and 8). The decline in cyclic AMP concentration that occurs after 20 min of stimulation with a high dose of 5-hydroxytryptamine is more akin to the receptor desensitization described in other systems.

It has been reported that prolonged stimulation with 5-hydroxytryptamine can inactivate the Ca^{2+} -gating response of salivary glands to the hormone (Berridge & Fain, 1979). This inactivation was associated with the loss of membrane-bound phosphatidylinositol and could be reversed *in vitro* only if inositol were supplied to the glands during recovery. The cyclic AMP response to 5-hydroxytryptamine is not so inactivated, demonstrating that its coupling mechanism does not involve phosphatidylinositol. The rapid decline phase of the initial cyclic AMP response seems to be associated with the action of phosphodiesterase. In the presence of the phosphodiesterase inhibitor theophylline, the decline phase is abolished and cyclic AMP concentration rises to very high values. Since the initial rate of rise in cyclic AMP concentration is not steepened by phosphodiesterase inhibition, it seems likely that a key factor in the fast biphasic response is the activation of phosphodiesterase.

A similar activation seems to occur under Ca^{2+} -free conditions (Fig. 7). The abrupt fall in cyclic AMP concentration in the glands, which begins after about 18 s of stimulation, ceases abruptly by 40 s, suggesting that phosphodiesterase too may be dependent on Ca^{2+} and that under ' Ca^{2+} -free' conditions may be controlled by the rate of mobilization of endogenous stores of the cation as soon as the Ca^{2+} immediately bound to the enzyme has been dispersed during its activity.

There is some indirect evidence to suggest that the turnover of cyclic AMP in resting glands is low since treatment with 10 mM-theophylline produced little change in the intracellular concentration of cyclic AMP. The early cyclic AMP response in these salivary glands may thus result from the sequential activation of adenylate cyclase and phosphodiesterase. These preliminary studies suggest that

Ca²⁺ may play an important part in the differential activation of these two enzymes, but further studies are necessary to unravel the interactions that exist *in vivo*.

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