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THE ROLE OF 5-HYDROXYTRYPTAMINE AND CYCLIC AMP IN THE CONTROL OF FLUID SECRETION BY ISOLATED SALIVARY GLANDS

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INTRODUCTION

The discovery of adenosine 3′,5′-monophosphate (cyclic AMP) has provided a focal point for studies on the mode of action of hormones. Cyclic AMP seems to function as an 'intracellular second messenger' mediating the action of many different hormones (Sutherland, Øye & Butcher, 1965; Sutherland & Robison, 1966; Robison, Butcher & Sutherland, 1968). The primary hormonal message arriving at a cell is translated into a secondary message, represented by an increase in the intracellular level of cyclic AMP, which is then responsible for influencing the effector system. Some of the most compelling evidence for this hypothesis is that exogenous cyclic AMP has the same effect as certain hormones (Haynes, Koritz & Péron, 1959; Orloff & Handler, 1962; Bitensky & Burstein, 1965; Grantham & Burg, 1966; Novales & Davis, 1967). Cellular activity therefore appears to be regulated by the intracellular level of cyclic AMP.

The concentration of cyclic AMP in cells is determined by the balance which exists between the two competing processes of synthesis and degradation. Cyclic AMP is synthesized from ATP by an enzyme adenyl cyclase which is associated with the plasma membrane (Sutherland, Rall & Menon, 1962; Davoren, & Sutherland, 1963). The concept has developed that many hormones act by stimulating adenyl cyclase to synthesize cyclic AMP. The adenyl cyclase in membrane fractions of certain tissues will respond to hormones and competitors in the same way as the intact cells (Murad *et al.* 1962; Bär & Hechter, 1969). Another line of evidence is that an increase in cyclic AMP level coincides with or, in some cases, precedes the observed action of many different hormones (Butcher, *et al.* 1965; Handler *et al.* 1965; Robison *et al.* 1965; Abe *et al.* 1969).

When the hormonal stimulus is withdrawn, the cyclic AMP concentration is reduced to its unstimulated level by means of a specific phosphodiesterase which converts cyclic AMP to adenosine 5′-monophosphate (5′-AMP). In an unstimulated cell the phosphodiesterase dominates resulting in a low level of cyclic AMP; in a stimulated cell, however, the enhanced adenyl cyclase activity can overcome the

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phosphodiesterase resulting in an increase in intracellular concentration of cyclic AMP. The balance which exists between these two competing processes can be upset by methyl xanthines (theophylline and caffeine) which inhibit the phosphodiesterase (Sutherland & Rall, 1958; Butcher & Sutherland, 1962). The ability of methyl xanthines either to modify the responsiveness of cells to hormones or to stimulate cells directly has provided further circumstantial evidence implicating cyclic AMP in the mode of action of hormones.

The salivary glands of the blowfly are being used as a model system to study the mode of action of hormones. The structural simplicity of these glands (Oschman & Berridge, 1970) and the ease with which they can be manipulated *in vitro* provides many advantages for studying the sequence of events underlying hormone action. Previously it was shown that isolated salivary glands were sensitive to low concentrations of 5-hydroxytryptamine (5-HT) and that the same effects were produced by cyclic AMP (Berridge & Patel, 1968). In this paper further evidence is provided to implicate cyclic AMP in the mode of action of 5-HT.

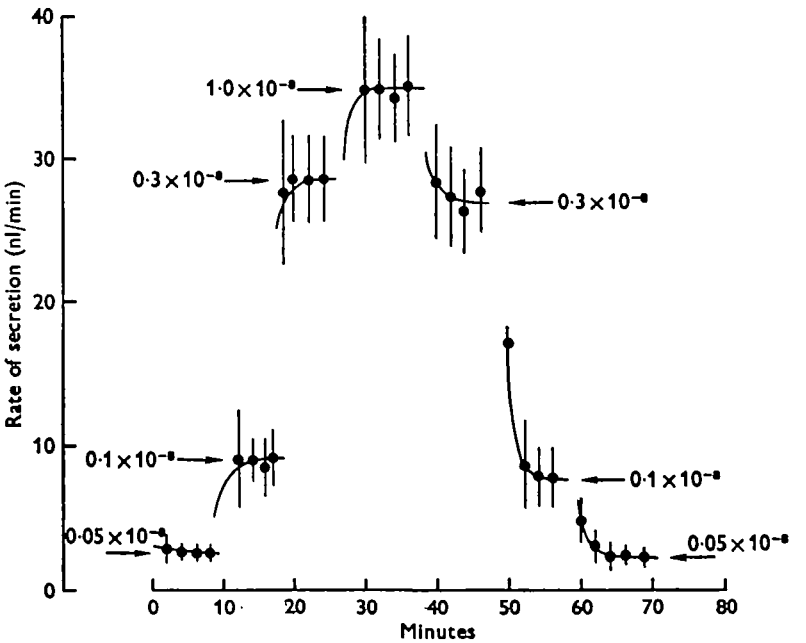

Fig. 1. The effect of different concentrations of 5-HT on rate of fluid secretion by isolated salivary glands. The response to any given concentration of 5-HT is independent of whether the concentration is being increased or decreased.

METHODS

The abdominal portions of the salivary glands of the adult blowfly *Calliphora erythrocephala* were isolated and set up in a defined bathing medium as described previously (Berridge & Patel, 1968).

Fine capillary pipettes were used to collect saliva and to change the bathing medium when different treatments were being applied. When a particular treatment was discontinued, salivary glands were washed three times with control medium to insure complete removal of the substance.

Dose-response curves were obtained by measuring the rate of secretion observed during a stepwise increase in the concentration of the stimulating molecule. One experiment used to test this method is shown in Fig. 1. The response of salivary glands to each concentration of 5-HT is the same irrespective of whether the 5-HT concentration is being increased or decreased. In this cumulative method the rate of secretion obtained for each concentration is expressed as a percentage of the maximum rate of secretion and plotted against the log of the concentration to give classical dose-response curves.

Cyclic AMP and N^6-2′-O-dibutyryl adenosine 3′,5′-monophosphate (dibutyryl cyclic AMP) were purchased from Sigma Chemical Co. The method described by Posternak, Sutherland & Henion (1962) was used to convert dibutyryl cyclic AMP to the N^6-monobutyryl derivative.

At least six salivary glands were used in each experiment and the vertical lines on the graphs represent twice the standard error of the mean.

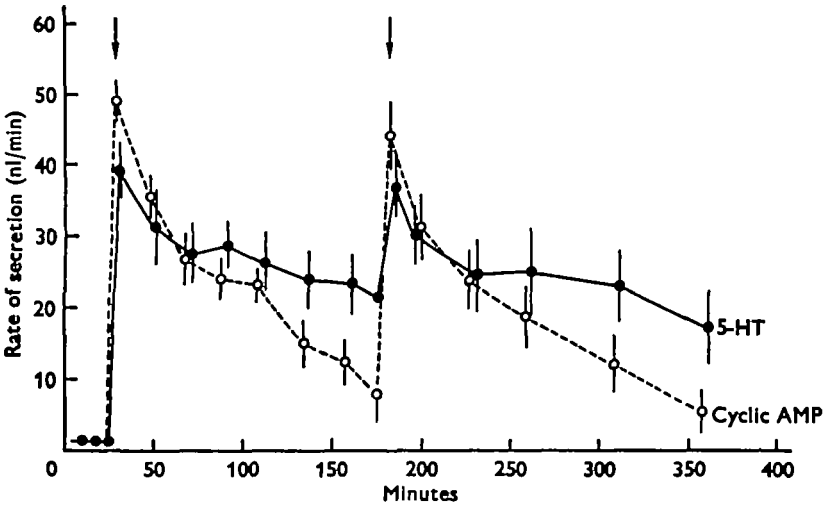

Fig. 2. The response of isolated salivary glands to 5-HT (5×10^{-8} M) and to cyclic AMP (1×10^{-3} M). These substances were added to salivary glands at 30 min. and fresh medium was added at 180 min. (arrows).

RESULTS

A. *The response of isolated salivary glands to 5-HT and cyclic AMP*

The first objective was to confirm that cyclic AMP could produce the same effects as 5-HT. Addition of 5-HT or cyclic AMP causes a large and sudden increase in the rate of secretion of isolated salivary glands (Fig. 2). Fluid was secreted at a relatively constant rate in the presence of 5-HT but declined regularly when salivary glands were stimulated with cyclic AMP. This decline could be temporarily reversed if the medium is replaced (Fig. 2), suggesting that it was due to a progressive change in the bathing medium rather than to any deterioration of the glands themselves. This was further tested by measuring the performance of isolated salivary glands when the bathing medium was frequently replaced (Fig. 3). In both cases rate of secretion remained much more constant and declined relatively little during the 6-hr observation period.

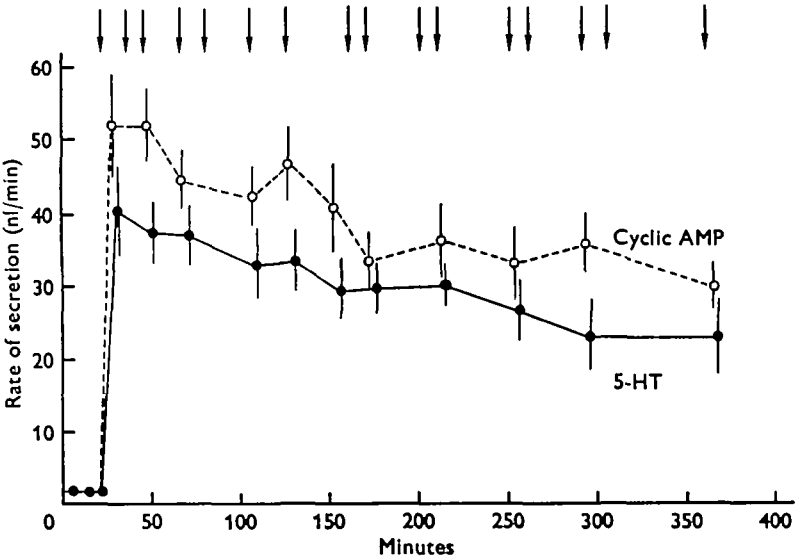

Fig. 3. The response of salivary glands to 5-HT (5×10^{-9} M) and to cyclic AMP (1×10^{-3} M) when the bathing medium was changed at frequent intervals (arrows).

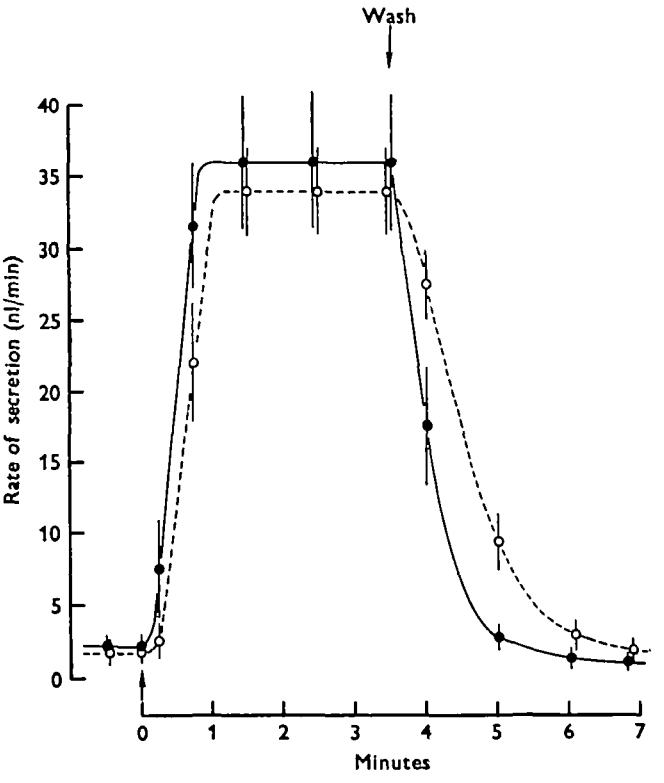

Fig. 4. The speed of response and recovery during brief stimulation with either 5-HT (5×10^{-9} M, ●) or cyclic AMP (1×10^{-3} M, ○). These substances were added at 0 min. (vertical arrow) and washed off at $3\frac{1}{2}$ min. (inverted arrow).

The next experiment compared the speed of response and the recovery time during a short period of stimulation with either 5-HT or cyclic AMP. In the case of 5-HT an increase in rate of fluid secretion is detectable within 30 sec. and a maximum rate of secretion occurs within 60 sec. (Fig. 4). The response to cyclic AMP is similar, but slightly slower. After removing 5-HT, the rate of secretion returns to the unstimulated rate in less than 60 sec., while recovery from stimulation with cyclic AMP takes slightly longer (Fig. 4). The rapid response and recovery of salivary glands enables

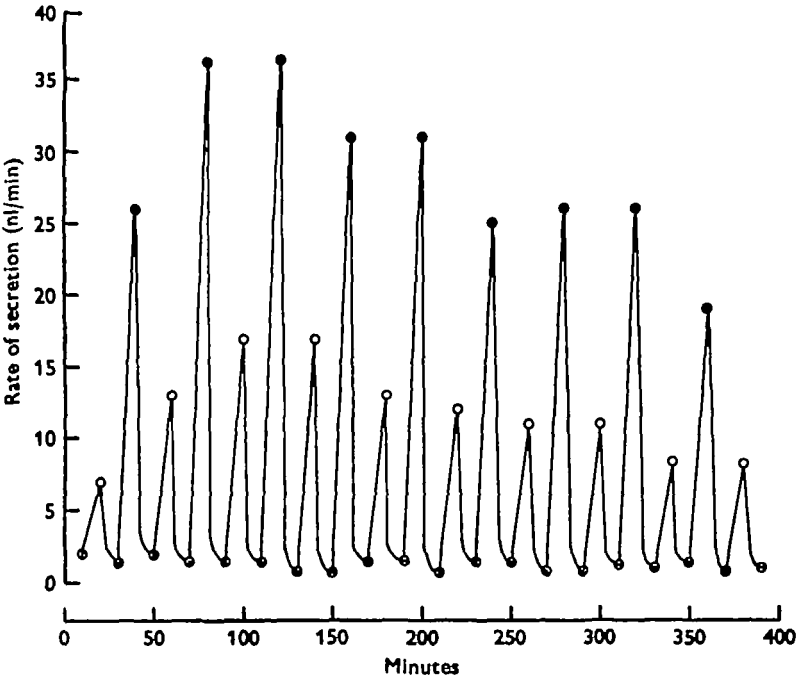

Fig. 5. The effect of repeated application of two different doses of 5-HT on rate of fluid secretion. ●, 3×10^{-6} M; ○, 2×10^{-6} M. After each application of 5-HT, rate of fluid secretion was determined in control medium (⊙).

one to test the effect of repeated stimulation with different doses of either 5-HT or cyclic AMP (Figs. 5, 6). In both cases the glands respond in a consistent way to the repeated alternate application of two different doses. The response of salivary glands to 5-HT or cyclic AMP therefore remains relatively constant irrespective of whether these compounds are applied continuously (Fig. 3) or intermittently (Figs. 5, 6).

B. *The effect of methyl xanthines*

The level of cyclic AMP in cells can be altered by interfering with the balance which exists between synthesis by adenyl cyclase and degradation by phosphodiesterase. Indirect evidence for the cyclic-AMP hypothesis can be obtained by studying the effects of methyl xanthines which inhibit phosphodiesterase. The first experiments showed that theophylline, but not caffeine, can stimulate fluid secretion (Fig. 7). However, even at high concentrations of theophylline (10 mM/l) the rate of secretion was much less than that obtained with either 5-HT or cyclic AMP. Furthermore, the time required to reach a maximum was very much longer than that for

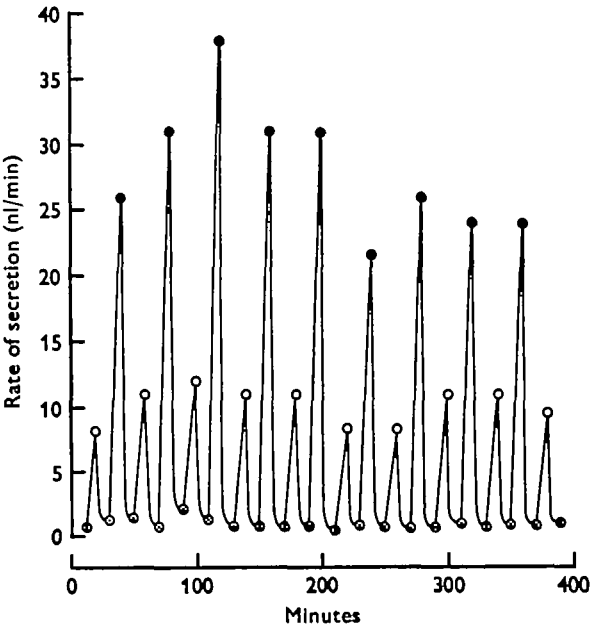

Fig. 6. The effect of repeated application of two different doses of cyclic AMP on rate of fluid secretion. ●, 4×10^{-3} M; ○, 3×10^{-3} M. After each application of cyclic AMP, rate of secretion was determined in control medium (⊙).

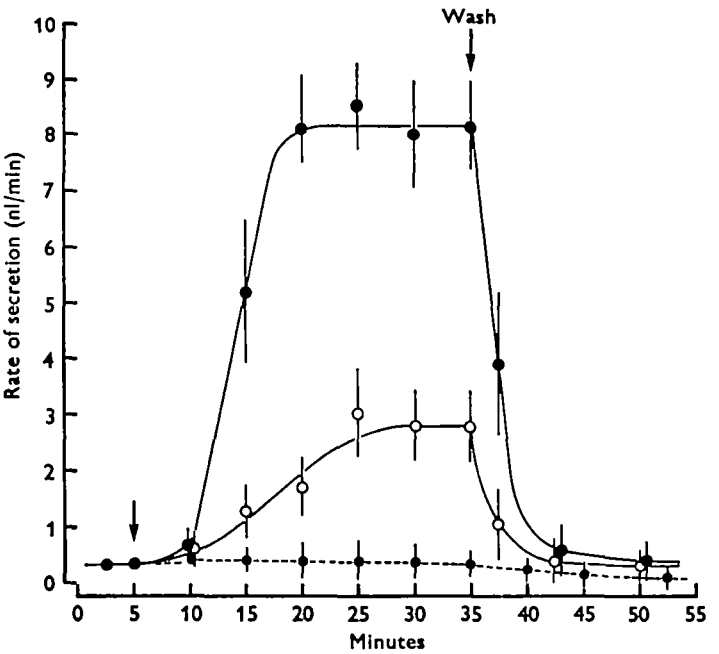

Fig. 7. Stimulation of fluid secretion by methyl xanthines. Theophylline (●——●, 10 mM/l; ○——○, 5 mM/l) and caffeine (●- - -●, 10 mM/l) were added at 5 min. (arrow) and washed off at 35 min. (arrow).

5-HT or cyclic AMP (Fig. 4). Rate of fluid secretion returned rapidly to the unstimulated level when theophylline was removed from the bathing medium (Fig. 7).

By reducing the rate at which cyclic AMP is degraded, methyl xanthines should be able to prolong the effect of 5-HT during recovery after washing. Salivary glands were

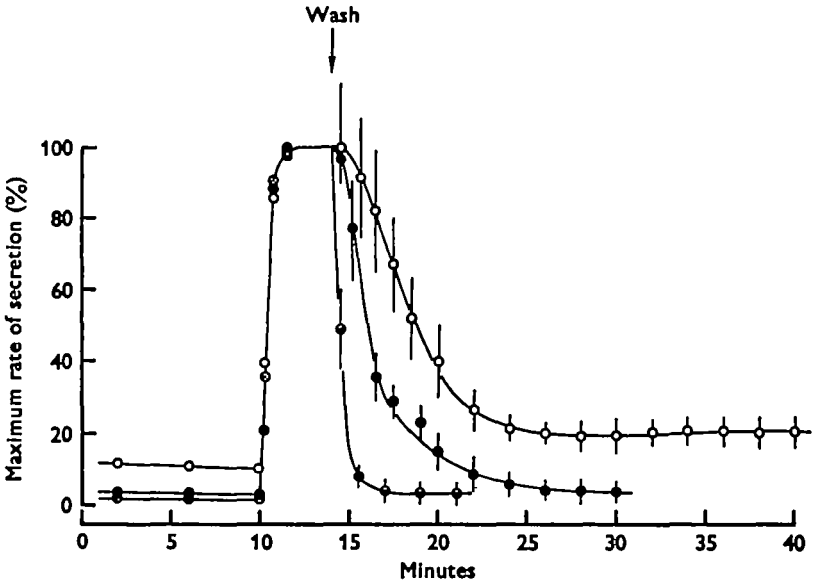

Fig. 8. The ability of theophylline to prolong the recovery time after removing 5-HT from the bathing medium. Isolated salivary glands were set up in artificial medium containing 5 mM/l (○) and 2 mM/l (●) theophylline. 5-HT was added at 10 min. and removed at 14 min. The control (⊙), which lacked theophylline, behaved as described earlier (Fig. 4).

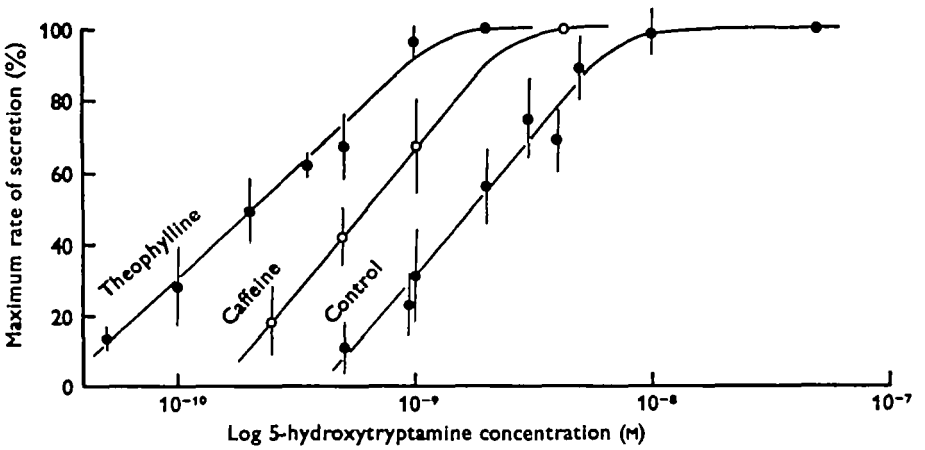

Fig. 9. The ability of theophylline (5 mM/l) and caffeine (10 mM/l) to sensitize salivary glands to 5-HT.

set up in two different concentrations of theophylline and allowed to secrete for 10 min. to establish a base line before stimulation with 5-HT. The time taken to reach a maximum rate of secretion was not affected but the recovery time after washing was greatly prolonged in those glands which were being treated with theophylline (Fig. 8).

Another effect of methyl xanthines is to sensitize salivary glands to either 5-HT or cyclic AMP as indicated by a marked shift to the left in the dose-response curves of these two substances. In the presence of theophylline or caffeine much lower doses of both 5-HT (Fig. 9) and cyclic AMP (Fig. 10) are capable of eliciting equivalent responses. In the case of 5-HT, the number of hormone-receptor interactions at these

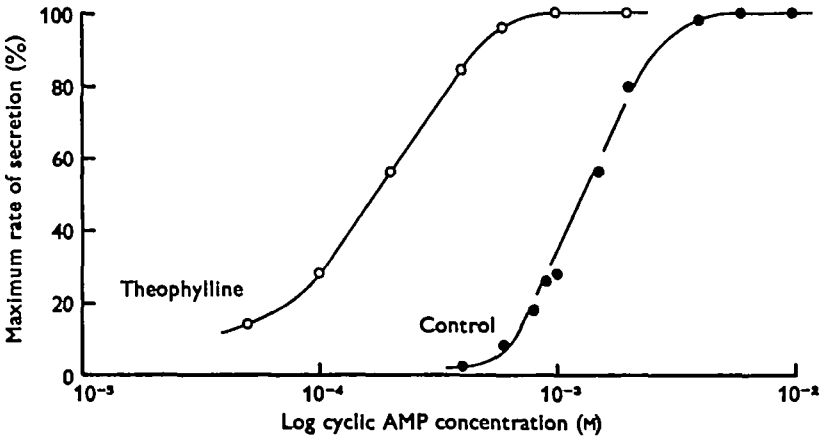

Fig. 10. The ability of theophylline (2 mM/l) to sensitize salivary glands to cyclic AMP.

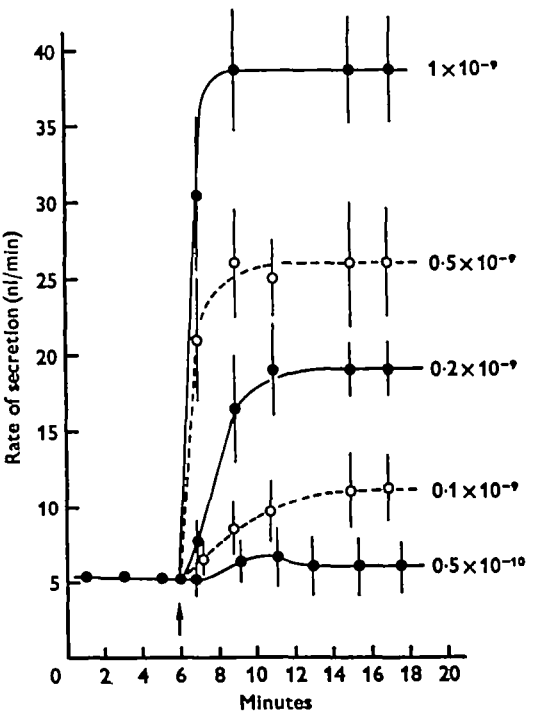

Fig. 11. The effect of theophylline on the speed of response of salivary glands to different concentrations of 5-HT. Salivary glands were set up in artificial medium containing theophylline (5 mM/l) and 5-HT was added at 6 min. (arrow). As the concentration of 5-HT decreases, the time required to reach a plateau increases.

lower doses must be decreased; the stimulation of adenyl cyclase and hence the synthesis of cyclic AMP should therefore be correspondingly slower. The time required to reach a maximum response when salivary glands are treated with different doses of 5-HT was determined in the presence or absence of theophylline. In the absence of theophylline, represented by the control dose-response curve in Fig. 9, a plateau was reached within 1 min. at all concentrations of 5-HT. However, when salivary glands were sensitized with 5 mM/l theophylline (Fig. 9), the time required to reach a plateau varies from 1 min. at high 5-HT concentrations to almost 10 min. at lower concentrations (Fig. 11).

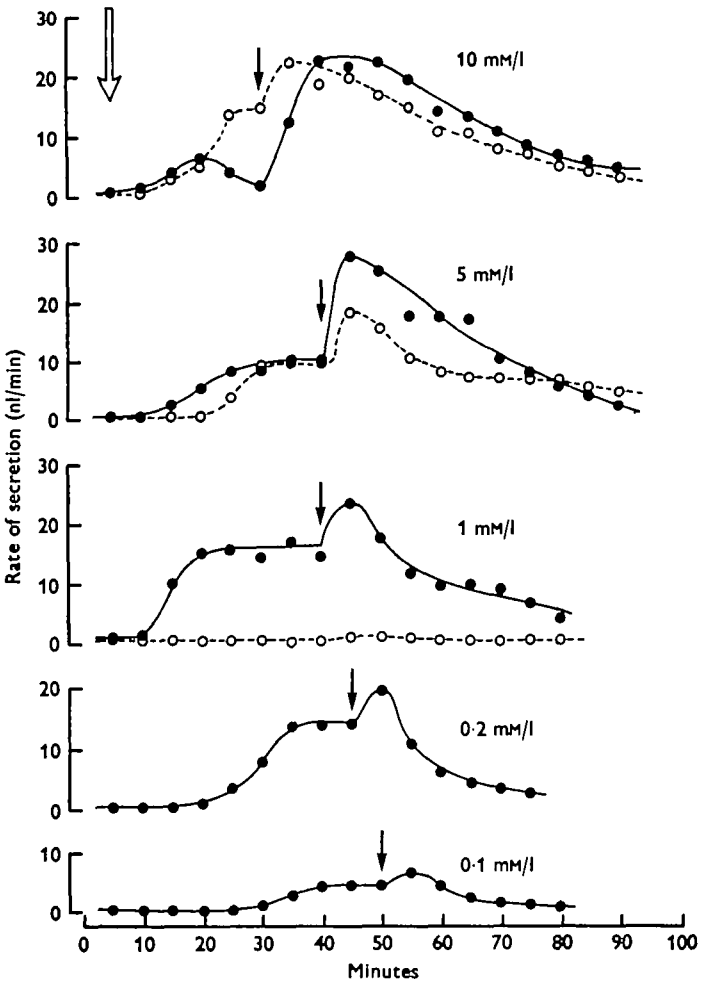

Fig. 12. The response of isolated salivary glands to different concentrations of N^6-monobutyryl cyclic AMP (●) and dibutyryl cyclic AMP (○). These derivatives were added at 5 min. (large arrow) and removed after various intervals (small arrows).

C. *The effect of N^6-monobutyryl and dibutyryl cyclic AMP*

The very high concentration of cyclic AMP needed to elicit a response (Fig. 10) probably depends on the low permeability of the outer cell membrane to this large molecule. Various derivatives of cyclic AMP have been prepared in an attempt to

increase its activity (Posternak *et al.* 1962; Falbriard, Posternak & Sutherland, 1967). When tested on salivary glands, the dibutyryl derivative was approximately equipotent, whereas N^6-monobutyryl cyclic AMP was more active than the parent compound. It is difficult to compare these compounds more precisely because the temporal response of salivary glands to these two derivatives (Fig. 12) is very different from that

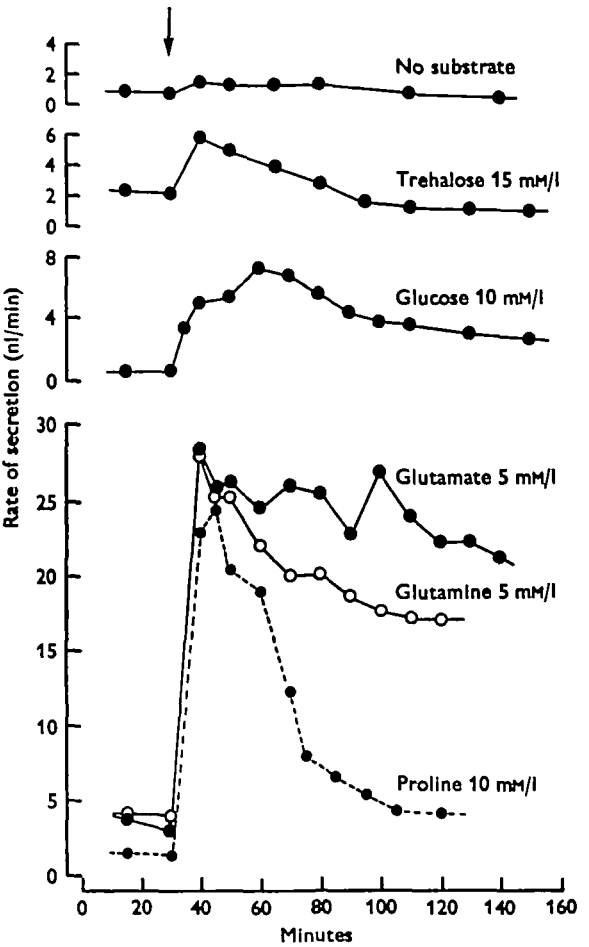

Fig. 13. The ability of different substrates to support fluid secretion during stimulation with 5-HT. Salivary glands were set up in media containing individual substrates and after 30 min. 5-HT was added (arrow).

to cyclic AMP (Fig. 4). Firstly, rate of secretion increases very slowly as compared to the rapid response to cyclic AMP. For example, salivary glands responded to 0·2 mM/l N^6-monobutyryl cyclic AMP after a lag period of more than 15 min. However, the response time was shortened appreciably as the concentration of these derivatives was increased. Secondly, the recovery time after washing off these substituted derivatives is also very much longer than that observed earlier for cyclic AMP (Fig. 4). Removal of these substances from the bathing medium was also accompanied by a sudden increase in rate of secretion (Fig. 12). In the case of N^6-monobutyryl cyclic AMP, the increase in rate of secretion after washing was larger at the higher

concentrations. The sudden surge in fluid flow immediately after washing seemed to indicate that these derivatives were partially inhibiting the glands. The much slower rate of secretion produced by the higher concentrations of N^6-monobutyryl cyclic AMP (Fig. 12) may represent another expression of this inhibition. When salivary glands are stimulated with 5-HT in the presence of N^6-monobutyryl cyclic AMP the response was only 50% of that obtained later with 5-HT alone.

D. *The substrate-dependence of the 5-HT response*

In certain systems cyclic AMP exerts its effect by stimulating a rate-limiting metabolic process. Preliminary observations on salivary glands indicate that, if a rate-limiting metabolic step is involved, it is more likely to be associated with the citric acid cycle than with glycolysis. Salivary glands lack an endogenous supply of energy because in the absence of metabolic substrates there is no stimulation of secretion (Fig. 13). In the presence of trehalose or glucose, 5-HT induced a small response. Normal responses were obtained with certain amino acids (Fig. 13); glutamine and glutamate maintained a high rate of secretion, whereas the response to proline was biphasic. Of the organic acids tested, malate produced a normal response, but acetate aspartate, fumarate and citrate were much less effective.

DISCUSSION

The present results suggest that cyclic AMP is involved in the control of fluid secretion by 5-HT. As in other systems, cellular activity seems to be regulated by the intracellular level of cyclic AMP which is determined by the balance which exists between its synthesis by adenyl cyclase and its degradation by phosphodiesterase (Fig. 14). In an unstimulated condition the level of cyclic AMP is kept low by the phosphodiesterase. Any procedure which will raise the intracellular level of cyclic AMP will result in an increase in cellular activity. It is now apparent that many hormones act by stimulating adenyl cyclase to synthesize cyclic AMP (Robison *et al.* 1968). The primary hormonal message arriving at the cell is thus translated into an increase in the intracellular level of cyclic AMP. These general concepts seem to apply to the mode of action of 5-HT in the salivary gland because all procedures designed to increase the intracellular level of cyclic AMP will accelerate fluid secretion.

The simplest method of raising the intracellular level of cyclic AMP is to add this compound to the bathing medium. All the experiments performed so far indicate that exogenous cyclic AMP reproduces all the effects of 5-HT on the rate of fluid secretion. The ionic composition of the saliva secreted during stimulation with either 5-HT or cyclic AMP is also identical (Oschman & Berridge, 1970). The very high concentration of cyclic AMP needed to stimulate secretion probably depends on a low permeability of the basal plasma membrane to this large negatively charged molecule. High concentrations of cyclic AMP are also required to reproduce the effects of melanophore-stimulating hormone (Bitensky & Burstein, 1965; Novales & Davis, 1967), vasopressin (Orloff & Handler, 1962; Grantham & Burg, 1966) and adrenocorticotropic hormone (Haynes *et al.* 1959; Imura *et al.* 1965). In certain systems the more lipid soluble dibutyryl analogue of cyclic AMP is much more effective than the parent compound (Bdolah & Schramm, 1965; Butcher *et al.* 1965; Imura *et al.*

1965; Pastan, 1966; Babad *et al.* 1967). The reason for this enhanced potency is not clear because the introduction of substituents may alter many of the parameters involved in the mode of action of cyclic AMP. For example, the increased lipid solubility of these butyryl derivatives may facilitate their entry into cells. On the other hand, the presence of substituents may reduce the effectiveness of cyclic AMP at its site of action with the effector system. There is some indication that the potency of cyclic AMP may depend on having a free hydroxyl group at the 2'-position (Fig. 14)

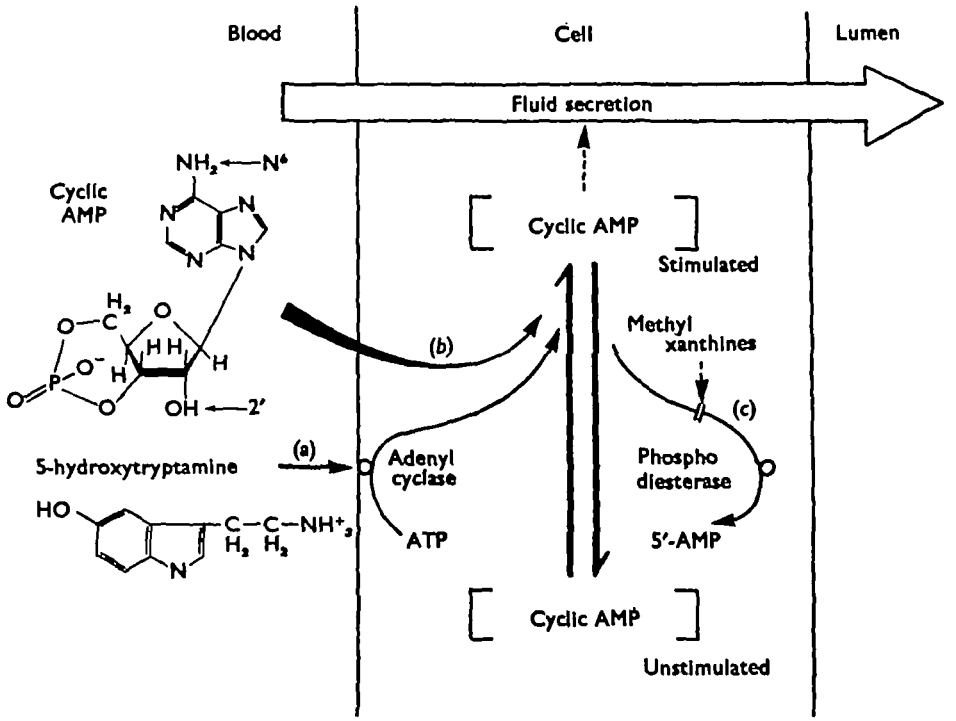

Fig. 14. Application of the cyclic AMP hypothesis (Orloff & Handler, 1962; Sutherland & Robison, 1966) to control of fluid secretion by salivary glands. 5-HT probably acts by stimulating adenyl cyclase to synthesize cyclic AMP from ATP (*a*); the increased intracellular level of cyclic AMP is then responsible for stimulating secretion. The intracellular level of cyclic AMP can be elevated artificially by adding this compound to the bathing medium (*b*), or by treatment with methyl xanthines which inhibit the phosphodiesterase which degrades cyclic AMP to 5'-AMP (*c*). Both these procedures will stimulate secretion. The small arrows on the cyclic AMP molecule represent the points where the two butyric acid chains are attached to form dibutyryl cyclic AMP (Falbriard *et al.* 1967).

on the ribose ring (Henion, Sutherland & Posternak, 1967). One possible explanation for the partial inhibition of 5-HT by N^6-monobutyryl cyclic AMP is competition with endogenous cyclic AMP at its site of action on the effector system. Finally, phosphodiesterase may not be capable of degrading these butyryl derivatives as effectively as cyclic AMP (Posternak *et al.* 1962). In the light of all these possibilities, the responses of salivary glands to N^6-monobutyryl and dibutyryl cyclic AMP are difficult to interpret. Since the response time is greatly increased, it might be argued that the cells are less permeable to these butyryl derivatives. However, the long lag

time can be explained equally as well by the time required to hydrolyse off the butyric acid substituents. The reason for the prolonged recovery time is equally uncertain. One possibility is that these butyryl derivatives may be resistant to phosphodiesterase as suggested by Posternak *et al.* (1962). Alternatively, these derivatives may act like methyl xanthines and prolong recovery by inhibiting phosphodiesterase.

The intracellular level of cyclic AMP can also be elevated by inhibiting the phosphodiesterase which is responsible for hydrolysing cyclic AMP to 5'-AMP (Fig. 14). Methyl xanthines, which are potent inhibitors of phosphodiesterase (Butcher & Sutherland, 1962), are capable of stimulating fluid secretion. Within the context of the model outlined in Fig. 14, this probably implies that in an unstimulated cell there is a slow, but continuous, endogenous synthesis of cyclic AMP. The level of cyclic AMP is kept low by the activity of the phosphodiesterase. By inhibiting this phosphodiesterase, however, the slow synthesis of cyclic AMP can raise the intracellular level sufficiently to stimulate secretion. The slow acceleration of fluid secretion by theophylline (Fig. 7) may reflect this gradual increase in intracellular concentration of cyclic AMP. The maximum stimulation obtained with theophylline, is somewhat less than that obtained with 5-HT or cyclic AMP, implying that the inhibition of phosphodiesterase was incomplete.

The nature of this unstimulated synthesis of cyclic AMP is unknown. The most likely explanation is that it represents a partial stimulation of the same adenyl cyclase which is sensitive to 5-HT. A successful 5-HT-receptor interaction seems to involve neutralization of an anionic site on the receptor by the positively charged quaternary nitrogen of 5-HT (unpublished observation). It is conceivable, therefore, that the slow synthesis of cyclic AMP in an unstimulated cell may result from a random neutralization of these anionic sites.

Methyl xanthines also have effects on salivary glands which are consistent with the model system outlined in Fig. 14. As one might expect, inhibiting the phosphodiesterase with theophylline will prolong the recovery time, as has been observed experimentally (Fig. 8). Under normal conditions when 5-HT is removed from the bathing medium the rate of secretion drops rapidly to the base line (Fig. 4). Presumably when the rapid production of cyclic AMP ceases, the phosphodiesterase can quickly reduce the cyclic AMP concentration to an unstimulated level. Methyl xanthines can also sensitize the cells to both 5-HT and cyclic AMP. A similar synergistic effect of theophylline has been found in other tissues (Rall & West, 1963; Butcher *et al.* 1965). By reducing the rate at which cyclic AMP is degraded, less cyclic AMP must be synthesized and thus fewer hormone-receptor interactions are necessary to stimulate cells. Thus, the responsiveness of cells to hormones may be regulated not only by varying the concentration of hormone, but also by adjusting the activity of the phosphodiesterase.

Although these studies with methyl xanthines strongly support the cyclic AMP-hypothesis, it is necessary to sound a cautionary note because it is well known that these purines may have effects on cells other than phosphodiesterase inhibition. In particular, caffeine can severely modify muscle contraction by interfering with intracellular calcium distribution (Sandow, 1965). Further studies will therefore be necessary to determine whether methyl xanthines influence fluid secretion by interfering with phosphodiesterase or by an indirect action possibly connected with calcium

metabolism. Keeping this reservation in mind, it is clear that all the evidence presented so far suggests that cyclic AMP may mediate the action of 5-HT as outlined in Fig. 14.

When 5-HT arrives at the cell it reacts with a specific receptor to stimulate the synthesis of cyclic AMP by adenyl cyclase. The resulting rise in intracellular cyclic AMP concentration is then responsible for stimulating fluid secretion. As the concept now stands, the sole effect of the hormone is to stimulate adenyl cyclase. However, in the case of biogenic amines like 5-HT it is conceivable that a successful 5-HT-receptor interaction may induce changes in cell function other than stimulation of adenyl cyclase. The exact mechanism whereby hormones activate the adenyl cyclase is unknown (Robison, Butcher and Sutherland, 1967). Another aspect to consider is that the adenyl cyclase system may play a reciprocal role in activating the 5-HT receptor. Attachment of ATP to adenyl cyclase could prime the receptor which is then de-activated by interaction with 5-HT resulting in synthesis and release of cyclic AMP.

Very little is known about the way in which cyclic AMP mediates the action of different hormones. Studies on liver and adipose tissue indicate that cyclic AMP acts on various rate-limiting metabolic reactions (Robison *et al.* 1968). Although the preliminary experiments reported here shed some light on the metabolism of salivary glands, they provide little insight into the mode of action of cyclic AMP. Salivary glands apparently lack an endogenous supply of energy since fluid secretion depends on having metabolic substrates in the bathing medium. Those substrates which pass directly into the citric acid cycle (glutamine, glutamate and malate) were much more effective than those entering at the beginning of the glycolytic pathway (trehalose and glucose). Perhaps this is a metabolic adaptation designed to provide large quantities of energy during short periods of salivation. Such a mechanism would resemble that found in insect muscle which uses proline to provide a rapid burst of energy at the onset of flight (Bursell, 1963). The mitochondria of salivary glands (Oschman & Berridge, 1970) certainly resemble those found in blowfly sarcosomes (Smith, 1963) in that they have tightly packed cristae containing orientated perforations. Another possibility to consider is that cyclic AMP acts by altering certain rate-limiting steps directly associated with the fluid transport mechanism. In the toad bladder and collecting duct of the mammalian kidney cyclic AMP appears to act by increasing the permeability of the mucosal membrane to water (Orloff & Handler, 1967; Grantham & Burg, 1966). The clarification of how cyclic AMP mediates the action of a wide range of hormones is an intriguing and challenging task for the future.

SUMMARY

1. Isolated salivary glands of *Calliphora* have been used as a system on which to study the mode of action of a hormone.

2. Cyclic AMP, which is thought to mediate the action of many different hormones, can stimulate fluid secretion equally as well as 5-HT.

3. Methyl xanthines, which inhibit the hydrolysis of intracellular cyclic AMP by phosphodiesterase, can stimulate secretion and prolong recovery time after removal of 5-HT.

4. Methyl xanthines can sensitize salivary glands to both 5-HT and cyclic AMP.

5. Butyryl derivatives of cyclic AMP can stimulate secretion, but their effect is slower and lasts longer.

6. Certain amino acids and malate support fluid secretion more effectively than trehalose or glucose.

7. It is concluded that cyclic AMP plays an important role in the ability of 5-HT to control fluid secretion by salivary glands.

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