J. Exp. Biol. (1973), **59**, 595–606
With 10 text-figures
Printed in Great Britain

THE EFFECTS OF DERIVATIVES OF ADENOSINE 3′,5′-MONOPHOSPHATE ON FLUID SECRETION BY THE SALIVARY GLANDS OF *CALLIPHORA*

BY M. J. BERRIDGE

A.R.C. Unit of Invertebrate Chemistry & Physiology, Department of Zoology, Downing Street, Cambridge

(*Received* 10 *April* 1973)

INTRODUCTION

Fluid secretion by the salivary glands of the blowfly *Calliphora* is regulated by 5-hydroxytryptamine (5-HT) (Berridge, 1970, 1972; Berridge & Prince, 1972). The action of 5-HT is apparently mediated by adenosine 3′,5′-monophosphate (cyclic AMP) which thus functions as an intracellular 'second messenger' as has been postulated in many other systems (Robison, Butcher & Sutherland, 1968). Exogenous cyclic AMP can reproduce the action of 5-HT on fluid secretion (Berridge & Patel, 1968; Berridge, 1970). The phosphodiesterase inhibitor theophylline is capable of stimulating secretion and can also sensitize salivary glands to both 5-HT and cyclic AMP (Berridge, 1970). Finally, the intracellular concentration of cyclic AMP is elevated during the action of 5-HT (Prince, Berridge & Rasmussen, 1972). An analysis of the electrical events associated with the actions of both 5-HT and cyclic AMP suggest that the latter functions by stimulating potassium transport (Berridge & Prince, 1972; Prince & Berridge, 1972). The specificity of this cyclic AMP-sensitive transport site has been analysed by testing the effects of a wide range of analogues closely related to cyclic AMP.

MATERIAL AND METHODS

Isolated salivery glands of adult female *Calliphora erythrocephala* were set up for the measurement of secretory rates as described previously (Berridge & Patel, 1968). The bathing medium used throughout these studies had the following composition (mM/l): NaCl, 120; KCl, 20; NaH_2PO_4, 8; $CaCl_2$, 2; $MgCl_2$, 2; trehalose, 5; glucose, 5; glutamine, 2; sodium glutamate, 2; proline, 2; alanine, 2; glycine, 2; malic acid, 2; citric acid, 2; fumaric acid, 2. Phenol red (10 μM/l) provided a constant check on the pH which was adjusted to 7·0–7·2.

A standard procedure was used for screening different cyclic nucleotides. Salivary glands were treated with two different concentrations (1 and 10 mM/l) of the nucleotide for up to 1 h with frequent (every 5 min) changes of the bathing medium. If the nucleotide stimulated secretion, further studies were made to compare its speed of response and potency with those of cyclic AMP. Dose-response curves were prepared using the same procedure as described for 5-HT analogues (Berridge, 1972). If the nucleotide failed to stimulate secretion, the glands were treated with 10^{-8} M 5-HT

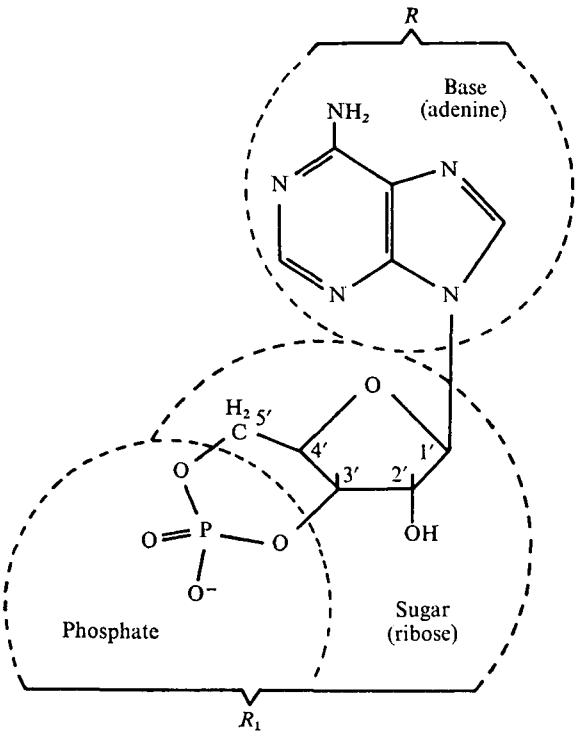


Fig. 1. The molecular structure of cyclic AMP. The two rigid ring systems R and R_1 are connected through a glycosidic bond. Most of the analogues tested had modification restricted either to the nitrogeneous base (R) or to the ribose and its cyclic phosphate group (R_1).

while still in the presence of the nucleotide. The subsequent response was compared with a control set of glands similarly treated with 5-HT to test for possible inhibitory effects of the cyclic nucleotide. The vertical lines on the graphs represent ± 2 S.E. of each mean ($n = 6$).

RESULTS

The cyclic AMP molecule (Fig. 1) consists of two rigid ring systems: the nitrogenous base adenine (R) is connected through a glycosidic bond to the sugar ribose which has a phosphate ring connected to its 3′- and 5′-carbon atoms (R_1). The molecular requirements and specificity of the cyclic AMP-receptor interaction was analysed by testing the effects of analogues containing modifications of all three regions.

(1) *The effect of altering the ribose or phosphate rings*

A number of compounds containing modifications of the ribose or phosphate ring (i.e. region R_1 in Fig. 1) were inactive both as agonists and antagonists (Table 1). The structural modifications of some of these inactive molecules are outlined below:

(*a*) Adenosine 5′-monophosphate and adenosine 3′-monophosphate; the phosphate ring has been opened at the 5′- and 3′-positions respectively (Fig. 1).

Table 1.

The following nucleotides, which differ from cyclic AMP by having modifications of the sugar or phosphate ring, did not stimulate salivary glands even when applied for 1 h at 10 mM/l. (*R* is equivalent to the base adenine as in Fig. 1).

Adenosine 5′-triphosphate (ATP)

Adenosine 5′-diphosphate (ADP)

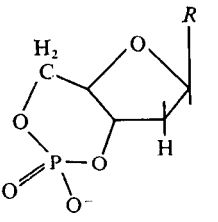
Adenosine 5′-monophosphate (AMP)

Adenosine 3′-monophosphate

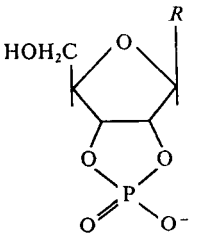
Adenosine 2′-monophosphate

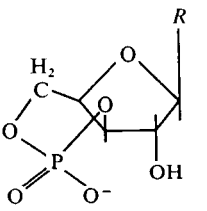
Adenosine 2′,3′-monophosphate

Desoxy-adenosine 3′,5′-monophosphate



Adenine 9-β-D-xylofuranosyl 3′,5′-monophosphate





(*b*) Adenosine 2′,3′-monophosphate; the phosphate ring is formed between the 2′- and 3′-positions of ribose (Table 1) rather than at the 3′- and 5′-positions as in cyclic AMP (Fig. 1).

(*c*) Desoxy-adenosine 3′,5′-monophosphate; the hydroxyl group at the 2′-position of ribose is replaced with a hydrogen atom (Table 1).

(*d*) Adenine 9-β-D-xylofuranosyl 3′,5′-monophosphate; the phosphate group is displaced above the plane of the xylofuranosyl ring through the formation of an unstrained diester linkage with the 3′-hydroxyl group (Table 1).

The results obtained with adenosine 5′-monophosphate (AMP) serve to illustrate the inactivity of these compounds (Fig. 2). There was no response to 10 mM/l AMP and the subsequent response and recovery to a 10-min treatment with 5-HT was no different from that of control glands. The only compound to show some competitive activity was adenosine 3′,5′-phosphorothioate where the double-bond oxygen atom is replaced with a bulkier sulphur atom. In the presence of this compound the response of salivary glands to 5-HT was much lower than that observed subsequently when the nucleotide was absent (Fig. 3*a*). Adenosine 3′,5′-phosphorothioate was also capable of partially blocking the stimulatory action of cyclic AMP (Fig. 3*b*).

Previous studies have shown that introduction of a butyryl group on the N^6-nitrogen of adenine considerably altered the activity of cyclic AMP (Berridge, 1970). A qualitatively similar response was obtained with N^6-benzoyl-adenosine 3′,5′-monophosphate (Fig. 4). The stimulation of secretion developed very slowly and was incomplete (approximately 25% of that obtained with 5-HT). On washing off N^6-benzoyl-adenosine 3′,5′-monophosphate there was a sudden increase in fluid secretion followed by a gradual return to the unstimulated level.

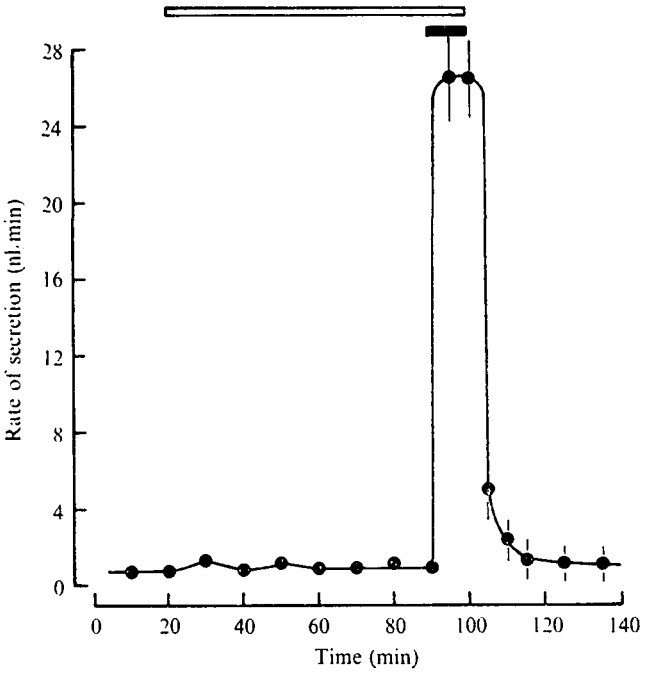


Fig. 2. The effect of 10^{-2} M/AMP (open bar) on rate of secretion by isolated salivary glands. Addition of 10^{-8} M 5-HT (solid bar) in the presence of AMP induced a normal increase in secretion.

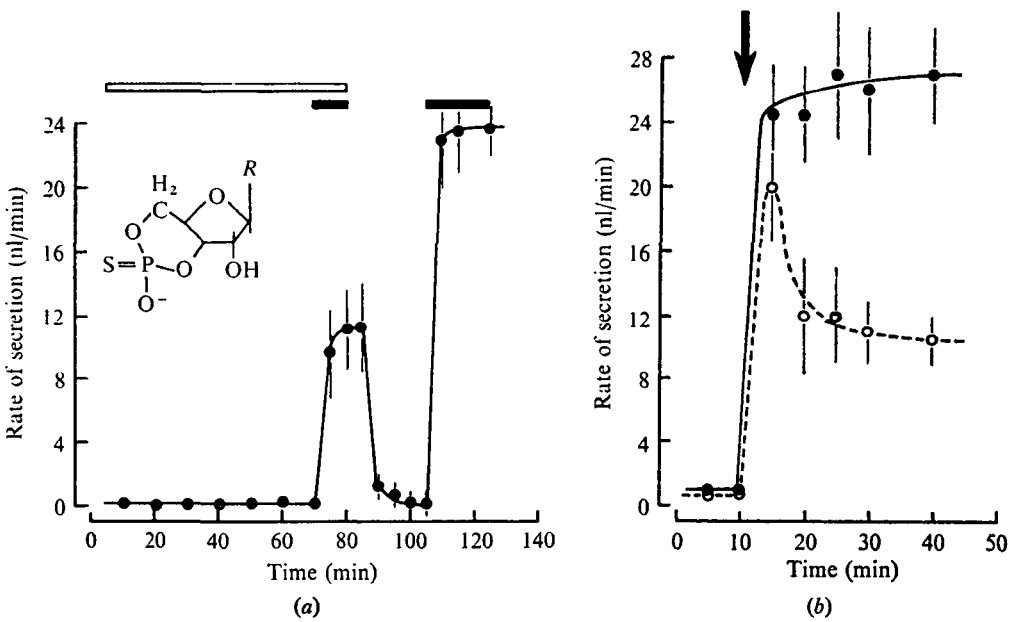


Fig. 3. The effect of adenosine 3′,5′-phosphorothioate. (*a*) The response to 10^{-8} M 5-HT (solid bars) was less in the presence of adenosine 3′,5′-phosphorothioate (open bar) than in its absence. (*b*) The response of isolated salivary glands to the addition (arrow) of 5 mM cyclic AMP in the presence (O – – O) or absence (●——●) of 5 mM adenosine 3′,5′-phosphorothioate.

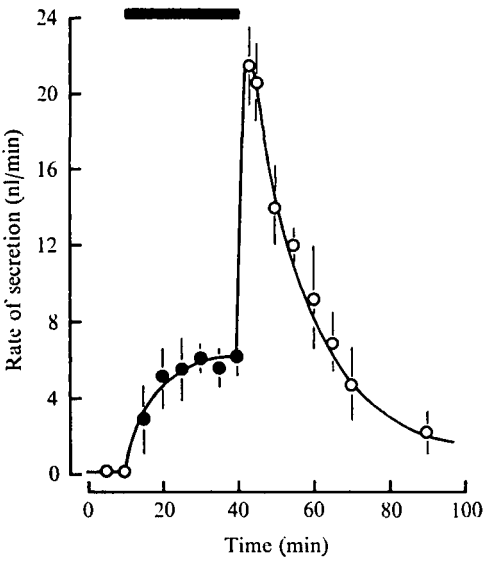


Fig. 4. The response of isolated salivary glands to a 30-min treatment (bar) with N^6-benzoyl-adenosine 3′,5′-monophosphate.

(2) *The effect of replacing adenine with different bases*

In the next series of experiments various compounds were tested which had modifications restricted to the base region of the molecule (Fig. 1, *R*). A few of the molecules also had substituents on the 2′-position of the ribose ring.

Guanine. Guanosine 3′,5′-monophosphate (cyclic GMP), in which adenine has been replaced with guanine, was totally inactive both as an agonist and antagonist. Dibutyryl cyclic GMP was also without effect.

Hypoxanthine. Inosine 3′,5′-monophosphate (cyclic IMP), in which adenine is replaced by hypoxanthine, can stimulate secretion but its effect was less than that observed with cyclic AMP (Fig. 5). The response took longer to develop in the case of cyclic IMP and the maximal rate of secretion was considerably less than that observed with cyclic AMP. After removal of these two nucleotides the rate of secretion declined more slowly in those glands which had been treated with cyclic IMP. There was also a brief increase in rate of secretion immediately after washing off cyclic IMP (Fig. 5).

Introduction of a butyryl substituent at the 2′-position of the ribose ring decreases the activity of cyclic IMP. Recovery after stimulation with 2′-*O*-butyryl-inosine 3′,5′-monophosphate was considerably slower than after cyclic IMP.

Tubercidin. The most active compound tested in the present series was tubercidin 3′,5′-monophosphate (cyclic TuMP) which was approximately ten times more active than cyclic AMP (Fig. 6). The onset of secretion was similar for both compounds but, in the case of cyclic TuMP, the response was slightly prolonged after washing off these two compounds (Fig. 7).

Uracil. The purine adenine could be replaced with the pyrimidine uracil with little alteration in activity. Uridine 3′,5′-monophosphate (cyclic UMP) was slightly more active than cyclic AMP (Fig. 8). The action of cyclic UMP resembled AMP

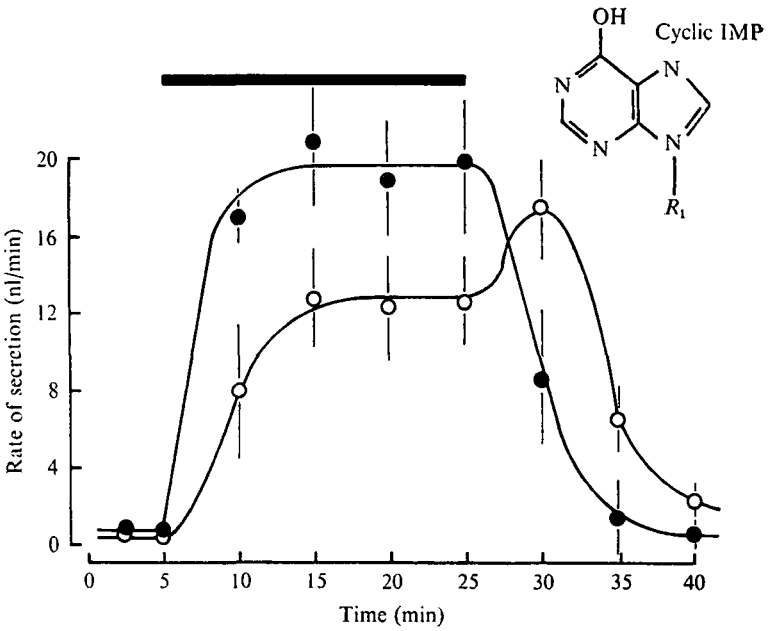


Fig. 5. Comparison of the effects of treating isolated salivary glands for 20 min (bar) with 10 mM cyclic AMP (●——●) or 10 mM cyclic IMP (○——○).

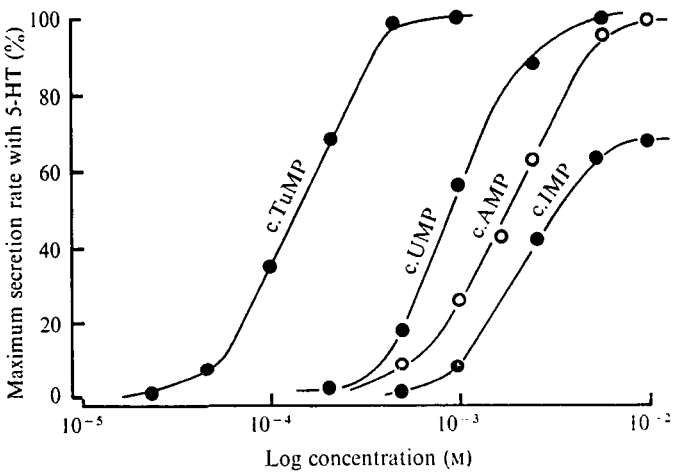


Fig. 6. Dose-response curves of different cyclic nucleotides. c.TuMP; tubercidin 3′,5′-monophosphate. c.UMP; uridine 3′,5′-monophosphate. c.AMP; cyclic AMP. c.IMP; inosine 3′,5′-monophosphate.

with regard to the onset of the response, but not with respect to recovery (Fig. 8). After removing cyclic UMP the rate of secretion returned slowly to the unstimulated level.

Cytosine. Treatment of salivary glands with cytidine 3′,5′-monophosphate (cyclic CMP), in which adenine is replaced with the pyrimidine cytosine, produced a partial and very gradual stimulation of secretion. An increase in secretion was noticeable

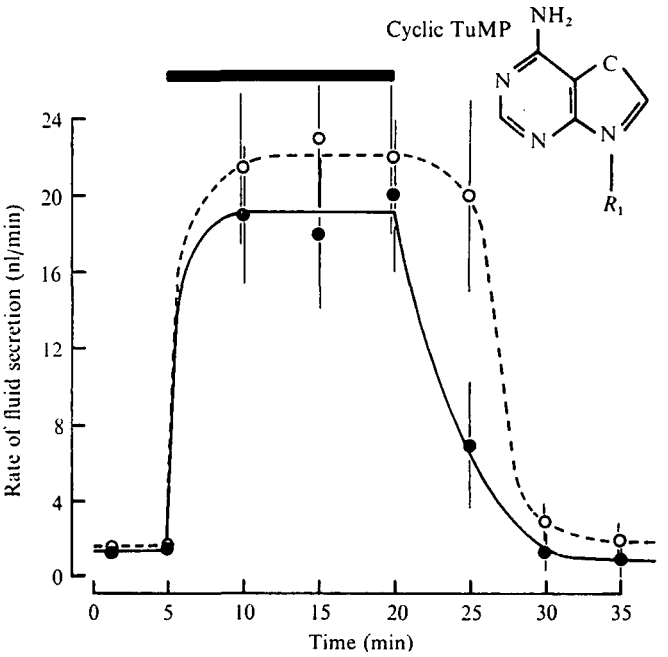


Fig. 7. Comparison of the effects of treating isolated salivary glands for 15 min (bar) with either 5 mM cyclic AMP (●——●) or 1 mM cyclic TuMP (○——○).

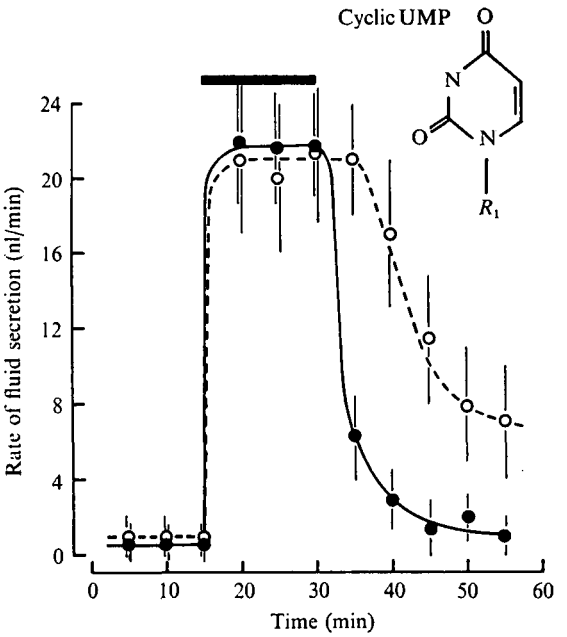


Fig. 8. Comparison of the effects of treating isolated salivary glands for 15 min (bar) with either 10 mM cyclic AMP (●——●) or 10 mM cyclic UMP (○——○).

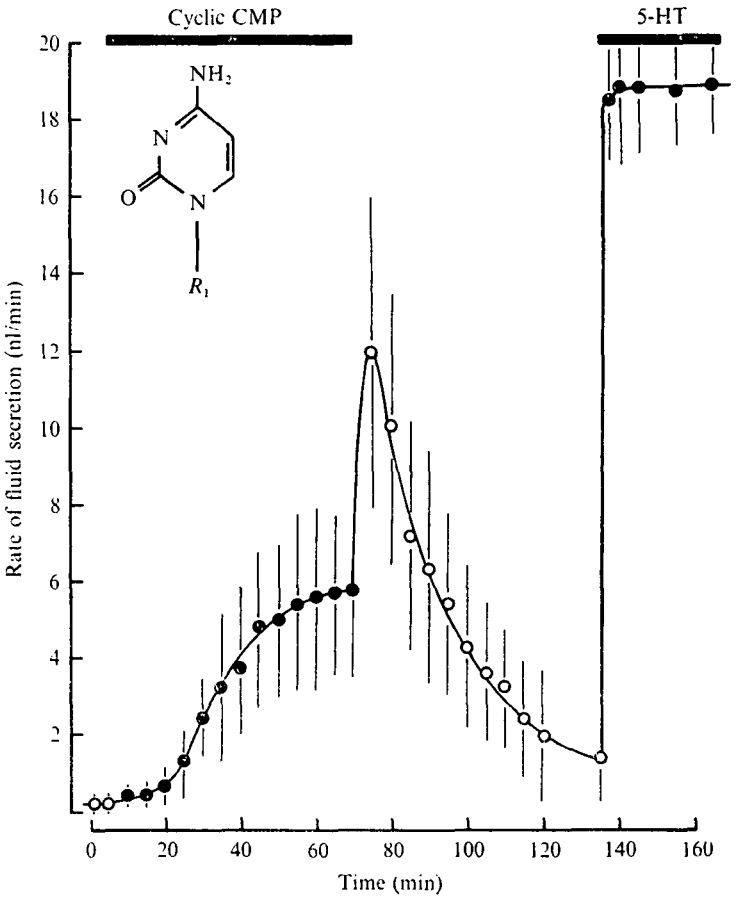


Fig. 9. The effect of treating isolated salivary glands for 65 min with 10 mM cyclic CMP. After washing the glands with control medium for a further 65 min they were treated with 10^{-8} M 5-HT.

about 15 min after addition of cyclic CMP and reached a plateau in 50–60 min. After washing off cyclic CMP the rate of secretion suddenly increased and then returned slowly to the unstimulated level (Fig. 9). The nature of the response is qualitatively similar to that produced by N^6-benzoyl-adenosine 3′,5′-monophosphate (Fig. 4). Cytidine 5′-monophosphate had no effect either as an agonist or antagonist.

The low activity of cyclic CMP was not caused by any non-specific inhibitory effect on cell function because normal high rates of secretion were observed if 5-HT was applied to salivary glands in the presence of cyclic CMP even after a prolonged treatment with the latter.

Thymine. Deoxythymidine 3′,5′-monophosphate had no effect on isolated salivary glands. In this molecule thymine replaces adenine and the hydroxyl group is absent from the 2′-position of the ribose ring.

DISCUSSION

5-Hydroxytryptamine stimulates fluid secretion by acting on the enzyme adenyl cyclase to increase the synthesis of cyclic AMP (Berridge, 1970; Prince *et al.* 1972).

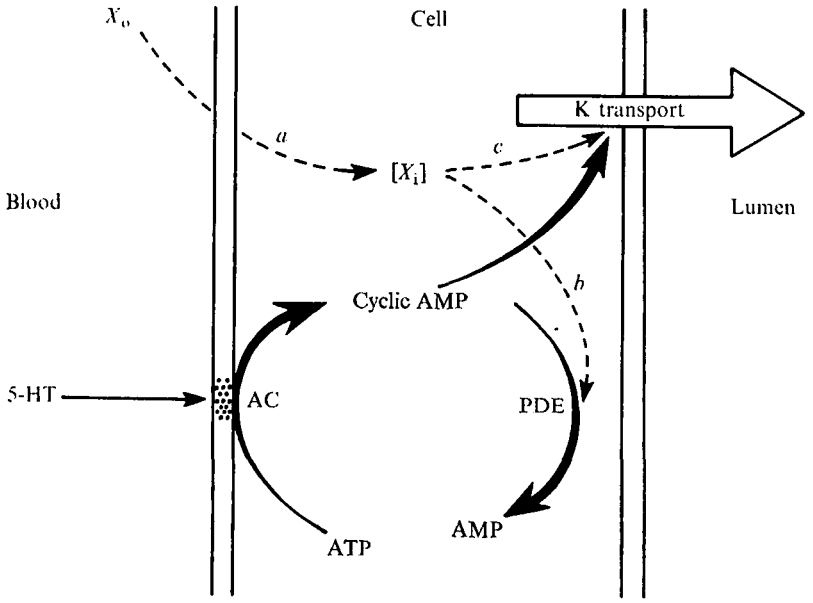


Fig. 10. A summary of the major factors determining the action of exogenous nucleotides. In order to simulate the action of endogenous cyclic AMP, which is normally increased when 5-HT activates adenyl cyclase (AC), an exogenous nucleotide (X_0) must penetrate the cell (*a*) fast enough to overcome possible degradation (*b*) by phosphodiesterase (PDE) before it can reach an internal concentration (X_1) which is high enough to activate potassium transport (*c*). See text for further details.

The increase in intracellular cyclic AMP concentration is then responsible for stimulating fluid secretion by increasing potassium transport across the cell (Berridge & Prince, 1972). Cyclic AMP is hydrolysed to adenosine 5′-monophosphate by a phosphodiesterase which can be inhibited by methyl xanthines such as theophylline (Berridge, 1970). The intracellular concentration of cyclic AMP is thus set by the balance which exists between synthesis by adenyl cyclase and degradation by phosphodiesterase (Fig. 10). On the basis of this model system (Fig. 10), there are three main factors which may influence the action of cyclic nucleotides when they are applied to intact cells.

First, there is the problem of entry into the cell (Fig. 10*a*); the very high concentration of cyclic AMP necessary to simulate the action of 5-HT probably results from a low permeability of the cell membranes to cyclic nucleotides (Berridge, 1970; Berridge & Prince, 1972). Indeed the ability of isolated salivary glands to secrete normally for over 6 h in a medium completely lacking in nucleotides attests to this low nucleotide permeability. Because of this penetration problem, it is difficult to assess the significance of negative results.

Second, once the nucleotide has entered the cell it may interact with the phosphodiesterase (Fig. 10*b*) and either be hydrolysed or, alternatively, it may inhibit this enzyme thus leading indirectly to an increase in the intracellular concentration of cyclic AMP. The latter phenomenon has been described in vertebrate adipose tissue where cyclic GMP stimulated lipolysis indirectly by increasing the intracellular level of cyclic AMP by inhibiting phosphodiesterase (Murad, Manganiello & Vaughan,

1970). The possibility of such indirect effects must be seriously considered in the case of *Calliphora* salivary glands because the phosphodiesterase inhibitor theophylline can stimulate secretion (Berridge, 1970). The very slow and incomplete stimulation of secretion observed with N^6-benzoyl-adenosine 3′,5′-monophosphate (Fig. 4) and cyclic CMP (Fig. 9) could be attributed to an increase in cyclic AMP concentration resulting from an inhibition of phosphodiesterase. The sudden increase in secretion which occurs when these two compounds are removed further complicates the interpretation of their mode of action. It is conceivable that such compounds have two actions within the cell; they may inhibit phosphodiesterase as well as compete with cyclic AMP at its site of action on the effector system. When these compounds are removed, a sudden surge in secretion would ensue if the inhibition on the action of cyclic AMP disappears sooner than the inhibitory effect on phosphodiesterase. Because it is difficult to interpret the action of such compounds which stimulate slowly and display complicated recoveries, they cannot be considered in the discussion of the structure-activity relationships of cyclic AMP.

The third, and most interesting site of action of cyclic nucleotides within the cell is on the effector system itself, in this case the process of potassium transport (Fig. 10*c*). Since the activity of the latter appears to be regulated by the internal concentration of cyclic AMP, it apparently possesses a cyclic AMP-sensitive site or 'receptor'. The structure-activity relationships described in this study provide some information on the molecular specificity of this receptor site. In this analysis, particular attention was paid to those compounds which stimulated fluid secretion with a time course similar to that of cyclic AMP. The relative activity of those nucleotides which could simulate the action of cyclic AMP are summarized on Fig. 6. The only chemical difference between these various active compounds was the nature of the base region of the molecule (i.e. *R* in Fig. 1). Since the receptor could accommodate different purine moieties (adenine, tubercidin, hypoxanthine) as well as the pyrimidine uracil, this region of the molecule appears to be relatively unspecific during the cyclic AMP-receptor interaction. The specificity of cyclic AMP apparently resides in the ribose and phosphate ring systems (Fig. 1, R_1), because any alterations in this region completely abolishes the activity of the molecule. This conclusion is subject to the reservation concerning entry into the cell mentioned earlier. However, it is unreasonable to conclude that all these inactive molecules were unable to enter the cell especially in view of the very high external concentrations applied to the cells. Of all the compounds tested, only adenosine 3′,5′-phosphorothioate displayed some competitive activity towards cyclic AMP. The phosphorothioate derivative may therefore interact with the receptor and thus partially inhibit the action of cyclic AMP. If, as seems likely, the negative charge located on the one phosphate oxygen atom is an important part of the cyclic AMP molecule, the presence of a bulky sulphur atom situated near this negative charge may introduce a steric hindrance preventing the latter from exerting its effect. Adenosine 3′-monophosphate, which is thought to inhibit the action of cyclic AMP in the exocrine pancreas (Kulka & Sternlicht, 1968), had no inhibitory effect on *Calliphora* salivary glands.

The lack of specificity for cyclic AMP in *Calliphora* salivary glands is consistent with the results of structure-activity studies on various vertebrate systems. Adipose and liver cells are sensitive to a range of cyclic nucleotides (Conn & Kipnis, 1969;

Blecher, Ro'Ane & Flynn, 1971). The site of action of cyclic AMP in heart, skeletal muscle and liver is thought to be the protein kinase which converts phosphorylase from its inactive to active form (Robison *et al.* 1968). A range of nucleotides have been tested on the isolated and partially purified protein kinases from various sources. Kuo & Greengard (1970) found that cyclic tubercidin 3′,5′-monophosphate could stimulate the protein kinase of bovine brain and heart as effectively as cyclic AMP. Working with the protein kinase isolated from bovine heart Drummond & Powell (1970) noted that cyclic tubercidin 3′,5′-monophosphate was also more active than cyclic AMP whereas adenine 9-β-D-xylofuranosyl-3′,5′-monophosphate and adenosine 3′,5′-phosphorothioate were inactive, which is consistent with the results on *Calliphora* salivary glands. A detailed study on the action of cyclic nucleotides on the activation of liver and muscle phosphorylase confirms the absence of specificity of the base region of the molecule (DuPlooy *et al.* 1971).

The pharmacological studies carried out so far thus indicate that ribose and the phosphate ring are of central importance during the action of cyclic AMP at its receptor site. Any alteration of these regions completely destroys or greatly reduces the activity of the molecule. The adenine base region of the molecule is apparently much less important since the various cyclic AMP-sensitive systems studied so far will accommodate a wide range of nucleotides containing substantial modifications in this region.

SUMMARY

1. The nature of the cyclic AMP-receptor interaction was analysed by testing a range of cyclic nucleotides on the isolated salivary glands of adult blowflies.

2. All compounds containing modifications in the region of ribose or the phosphate ring were inactive. One compound, adenosine 3′,5′-phosphorothioate, appeared to compete with cyclic AMP.

3. A number of nucleotides with alterations restricted to the base region of the molecule could stimulate secretion equally as well as cyclic AMP.

4. These observations indicate that during the action of cyclic AMP the phosphate ring and ribose sugar are critical whereas the adenine ring plays a relatively unspecific role.

I am most grateful to the following for gifts of compounds: adenosine 3′,5′-phosphorothioate, Dr F. Eckstein; tubercidin 3′,5′-monophosphate, Dr A. R. Hanze, The Upjohn Company; dibutyryl cyclic GMP and N^6-benzoyladenosine 3′,5′-monophosphate, Drs M. Nelboeck and G. Michal, Boehringer; 9-β-D-xylofuranosyl 3′,5′-monophosphate, The Cancer Chemotherapy National Service Center.

REFERENCES

BERRIDGE, M. J. (1970). The role of 5-hydroxytryptamine and cyclic AMP in the control of fluid secretion by isolated salivary glands. *J. exp. Biol.* **53**, 171–86.

BERRIDGE, M. J. (1972). The mode of action of 5-hydroxytryptamine. *J. exp. Biol.* **56**, 311–21.

BERRIDGE, M. J. & PATEL, N. G. (1968). Insect salivary glands: stimulation of fluid secretion by 5-hydroxytryptamine and adenosine 3′,5′-monophosphate. *Science, N.Y.* **162**, 462–3.

BERRIDGE, M. J. & PRINCE, W. T. (1972). The role of cyclic AMP and calcium in hormone action. *Adv. Insect Physiol.* **9**, 1–49.

BLECHER, M., RO'ANE, J. T. & FLYNN, P. D. (1971). Biological roles for 3',5'-cyclic nucleotides. I. Lipolytic agents in isolated rat epididymal adipose cells and substrates for adipose tissue phosphodiesterase. *Archs Biochem. Biophys.* **142**, 351–362.

CONN, H. O. & KIPNIS, D. M. (1969). The effects of various 3',5'-cyclic nucleotides on gluconeogenesis and glycogenolysis in the perfused rat liver. *Biochem. biophys. Res. Commun.* **37**, 319–26.

DRUMMOND, G. I. & POWELL, C. A. (1970). Analogues of adenosine 3',5'-cyclic phosphate as activators of phosphorylase *b* kinase and as substrates for cyclic 3',5'nucleotide phosphodiesterase. *Mol. Pharmacol.* **6**, 24–30.

DUPLOOY, M., MICHAL, G., WEIMANN, G., NELBOECK, M. & PAOLETTI, R. (1971). Cyclophosphates. I. Effect of various cyclophosphates on phosphorylase *b* kinase activation. *Biochim. biophys. Acta* **230**, 30–9.

KULKA, R. G. & STERNLICHT, E. (1968). Enzyme secretion in mouse pancreas mediated by adenosine-3',5'-cyclic phosphate and inhibited by adenosine-3'-phosphate. *Proc. Natn. Acad. Sci. U.S.A.* **61**, 1123–8.

KUO, J. F. & GREENGARD, P. (1970). Stimulation of adenosine 3',5'-monophosphate-dependent and guanosine 3',5'-monophosphate-dependent protein kinases by some analogs of adenosine 3',5'-monophosphate. *Biochem. biophys. Res. Commun.* **40**, 1032–8.

MURAD, F., MANGANIELLO, V. & VAUGHAN, M. (1970). Effects of guanosine 3',5'-monophosphate on glycerol production and accumulation of adenosine 3',5'monophosphate by fat cells. *J. biol. Chem.* **245**, 3352–60.

PRINCE, W. T. & BERRIDGE, M. J. (1972). The effects of 5-hydroxytryptamine and cyclic AMP on the potential profile across isolated salivary glands. *J. exp. Biol.* **56**, 323–33.

PRINCE, W. T., BERRIDGE, M. J. & RASMUSSEN, H. (1972). Role of calcium and adenosine-3',5'-cyclic monophosphate in controlling fly salivary gland secretion. *Proc. Natn. Acad. Sci. U.S.A.* **69**, 553–7.

ROBISON, G. A., BUTCHER, R. W. & SUTHERLAND, E. W. (1968). Cyclic AMP. *Ann. Rev. Biochem.* **37**, 149–74.