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Role of Calcium and Adenosine-3′:5′-Cyclic Monophosphate in Controlling Fly Salivary Gland Secretion

(membrane permeability/adenylate cyclase/blowfly/protein kinases)

WILLIAM T. PRINCE, MICHAEL J. BERRIDGE, AND HOWARD RASMUSSEN*

Agricultural Research Council, Unit of Invertebrate Chemistry and Physiology, Department of Zoology and Department of Pharmacology, Cambridge University, England

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ABSTRACT The action of 5-hydroxytryptamine (5-HT) on an insect salivary gland was associated with a rise in adenosine-3′:5′-cyclic monophosphate (cAMP) concentration and an increase in calcium uptake. An increase in secretion induced either by 5-HT or exogenous cAMP required extracellular calcium. Both 5-HT and exogenous cAMP increased ^{45}Ca efflux from previously labeled glands, but only 5-HT caused an increase in calcium uptake. The transepithelial potential in this tissue became more negative after addition of 5-HT, but more positive after addition of cAMP. 5-HT and cAMP induced a more negative potential when calcium was removed from the medium. It was concluded that both calcium and cAMP serve as intracellular messengers when 5-HT acts upon fly salivary gland.

When many different types of mammalian or amphibian cells are activated by specific extracellular stimuli, the concentration of adenosine-3′:5′-cyclic monophosphate (cAMP) within the cell increases (1, 2). These observations led Sutherland, Øye, and Butcher (3) to propose the second messenger concept of cell activation in which a specific first or extracellular messenger activates an adenylate cyclase in the plasma membrane of the receptive cell. The resultant increase in intracellular cAMP was considered to act as a second messenger that translates the extracellular message to cellular response. The proposed mode of action of the second messenger in animal cells is that of activating a class of enzymes known as protein kinases (4–6).

This simple model does not adequately account for a number of observations (8–16). An alternative model that includes both calcium and cAMP as mediators of cell response has been proposed (7, 8, 17); cell activation by the extracellular messenger leads both to an increase in calcium entry and an activation of adenylate cyclase. The subsequent rise in intracellular cAMP leads, in turn, to both an activation of protein kinases and to a redistribution of calcium from mitochondrial matrix space to cell cytosol. The increase in cytosolic calcium leads to an activation of calcium-dependent enzymes (12, 14), a change in permeability of the cell membrane to monovalent cation (18), and an inhibition of adenylate cyclase (19).

The support for this second model has been derived from studies with mammalian cells. However, cAMP has been found in cells of organisms from various phyla and genera (2, 20). Clearly, if a fundamental relationship does exist between calcium and cAMP, then evidence for this relationship should be found in animals other than mammals. In a search for such evidence, we have examined in detail a specific insect tissue, the salivary gland, because earlier studies (21, 22) suggested that cAMP probably served as a second messenger in its activation by 5-hydroxytryptamine (5-HT).

Our studies have included an analysis of the effects of 5-HT and exogenous cAMP upon secretion rate and transepithelial potential in salivary glands in the presence or absence of calcium and their effect upon ^{45}Ca uptake and efflux, and the effect of 5-HT upon tissue concentrations of cAMP in the presence or absence of calcium. Calcium ion was deleted in these experiments by removal of calcium and addition of a specific chelating agent, ethyl-eneglycol bis-(aminoethyl) tetraacetic acid (EGTA) to the incubation medium. Incubations were performed either at room temperature (24–25°) or at 27°.

Calcium and Secretion. When either 10 nM 5-HT or 10 mM cAMP was added to the medium bathing the isolated blowfly salivary gland, there was a prompt rise in the secretory rates from 0.7–0.8 nl/min to 40–50 nl/min (Fig. 1). This maximal rate was reached within 1–3 min, usually being attained more rapidly if induced by 5-HT than by cAMP (22). Once attained, the rate was maintained at nearly the same value as long as the 5-HT or cAMP was present. Upon their removal, the rate of secretion dropped to control value within a few minutes.

A different response was obtained when either 5-HT or cAMP was added to salivary glands in the absence of calcium (Fig. 1). When 5-HT was added, the rate of secretion rose rapidly to approximately two-thirds of its normal maximal value. It then declined gradually over the next 30 min to about 10% of the normal maximal rate. A change from the EGTA-containing to the calcium-containing medium (Fig. 1), both with 5-HT, led to a very rapid rise in the rate of secretion to normal maximal values within 1–2 min. This maximal rate was sustained for the next 15 min. The pattern of change after addition of cAMP to the EGTA–medium was qualitatively similar to that seen after addition of 5-HT, except for the time course of this change. Normally when 10 mM cAMP was added, the rate of secretion rose

Abbreviations: 5-HT, 5-hydroxytryptamine; EGTA, ethylene glycol bis-(amino-ethyl) tetraacetic acid.

* Present address: Department of Biochemistry, University of Pennsylvania, School of Medicine, Philadelphia, Pa. 19104. To whom reprint requests should be addressed.

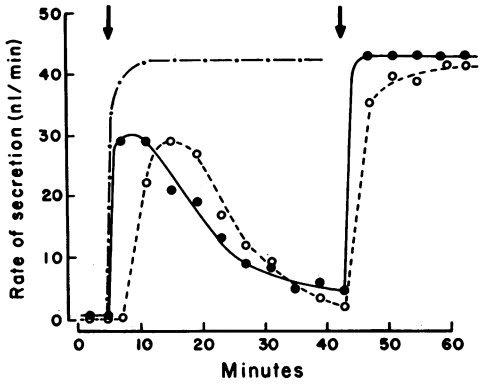

FIG. 1. Enhancement of the rate of fluid secretion in isolated salivary glands (*first arrow*) by 5-HT or cAMP in the presence or absence of calcium. In the presence of 2.0 mM calcium (—·—·), there is no difference in the action of 10 nM 5-HT or 10 mM cAMP (data taken from Berridge, ref. 22). In a calcium-free medium (containing 5 mM EGTA), the effects of 10 nM 5-HT (●——●) and 10 mM cAMP (O- - -O) are different. After 43 min of stimulation (*second arrow*), these calcium-free media were changed to ones containing the respective stimulant and 2.0 mM calcium. In addition to either 2.0 mM Ca^{2+} or 5 mM EGTA, the medium contained 155.5 mM sodium, 20 mM potassium, 156 mM chloride, 2 mM magnesium, 3.5 mM phosphate, 2.7 mM malate, 2.7 mM glutamate, 1.8 mM citrate, 10 mM glucose, and <0.01 mM phenol red. The pH was 7.2 ± 0.1. Incubations were performed at 24–25°.

promptly, but when the glands were incubated with cAMP in the EGTA–medium (Fig. 1), the rate of secretion rose slowly to two-thirds of the normal maximal value in about 10 min after which it fell to about 5% of the normal maximal rate in the next 25 min. A change in medium from one containing EGTA and cAMP to one containing 2.0 mM calcium and cAMP led to a rise in the rate of secretion to normal maximal values in 15 min, but this calcium-induced increase in secretory rate was slower than that detected when 5-HT was present (Fig. 1).

Calcium and cAMP Production. The change in cAMP concentration in response to 5-HT was measured by the method of Gilman (23) that uses a cAMP-binding protein and measures absolute concentrations. The concentration of cAMP in control glands was 0.06–0.1 pmol/gland (Fig. 2). This value did not vary significantly with time, nor was it changed when the glands were incubated in the absence of calcium. When 5-HT was added, in the presence of 0.5 mM calcium, the concentration of cAMP increased 3- to 4-fold within 2 min, then fell to approximately twice the control value ($P < 0.001$) at 6 min and remained at this concentration for at least 25 min (Fig. 2). When 5-HT was added, in the presence of 5 mM EGTA, the concentration of cAMP rose less rapidly, but eventually reached a higher plateau value than when calcium was present (Fig. 2). When measured 25 min after addition of 5-HT, the cAMP concentration was higher in glands incubated in the absence of calcium than in those incubated in the presence of calcium (Fig. 2). Thus, at a time when secretion was considerably greater in the presence than in the absence of calcium (Fig. 1), the concentration of cAMP was lower (Fig. 2), indicating that there was no direct correlation between the rate of secretion and concentration of cAMP in the calcium-deficient medium.

It was necessary to rule out the possibility that the measured changes in cAMP concentration were due to guanosine-3′:5′-cyclic monophosphate (cGMP) rather than cAMP itself because of recent data showing that cGMP concentrations in some insect tissues are 2–3 times higher than the concentration of cAMP (24). This possibility seemed unlikely in view of the fact that Gilman (23) had shown that the binding protein used in his assay had a 1000-fold lower affinity for cGMP than for cAMP. Nevertheless, to be quite certain, the effects of cGMP and other nucleotides upon secretion were examined; a particulate hormone-sensitive adenylate cyclase was looked for; and changes in cAMP concentrations were measured by an alternate technique (25).

No other nucleotide, including cGMP, induced secretion when added to the bath. Cyclic GMP had no effect even at a concentration of 10 mM. By use of a particulate preparation of fly salivary glands and standard methods for measurement of adenylate cyclase activity (26), NaF-sensitive adenylate cyclase was found; its activity was stimulated 3- to 4-fold by the addition of 10 nM 5-HT. Finally, fly salivary glands were incubated with 100 μCi of [^{3}H]adenine (8.4 mCi/mol), washed, and incubated with 10 mM theophylline to inhibit phosphodiesterase (1). The content of [^{3}H]cAMP was determined after 10-min incubation in the absence or presence of 50 nM 5-HT. In the presence of calcium, 5-HT caused an increase from 18 ± 2 to 161 ± 3 cpm/gland in the rate of [^{3}H]cAMP formation. In the absence of calcium, 5-HT caused an increase from 44 ± 4 to 163 ± 5 cpm/gland. The labeled compound formed under these conditions was subjected to chromatography in two different solvents known to separate cAMP from cGMP (26, 27). Over 90% of the radioactivity recovered behaved like cAMP.

Calcium and Transepithelial Potential. In previous work, techniques were developed for measurement of the potential across the salivary gland (28, 29). It was found that when 10 nM 5-HT stimulated secretion in the presence of 0.5 mM or higher concentration of calcium in the incubation medium,

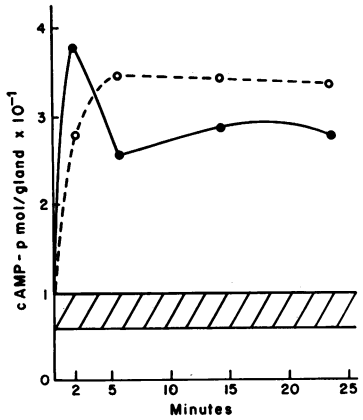

FIG. 2. The enhancement of the concentration of cAMP by 10 nM 5-HT in the blowfly salivary gland incubated at 27° in the absence (5 mM EGTA, O- - -O) and presence (0.5 mM, ●—●) of calcium. Cyclic AMP was measured by its ability to displace a standard amount of [^{3}H]cAMP from a cAMP-binding protein isolated from bovine skeletal muscle (6). The procedure followed was that described by Gilman (23), except for the preparation of tissue samples and standards. *Shaded area* represents the mean control values ± 2 SEM.

the luminal potential became more negative with respect to the bathing medium (28) (Fig. 3*a*). When 10 mM cAMP activated secretion, the potential became more positive (28) (Fig. 3*c*). When glands were incubated in medium with 5 mM EGTA (see legend of Fig. 1), the initial change in potential after the addition of 10 mM cAMP was similar to that observed in glands incubated in the presence of calcium (Fig. 3*d*). In contrast, the removal of calcium altered the response to 5-HT (Fig. 3*b*); after an initial increase in negative potential, it became more positive and similar in magnitude to that seen after addition of cAMP. When calcium was added again to the medium, the potential rapidly reverted to a negative value that was characteristic of the gland stimulated with 5-HT in the presence of calcium.

Calcium Uptake. The effect of both 5-HT and exogenous cAMP upon the accumulation of ^{45}Ca was examined by adding them to the glands 5 min before the addition of 10 μCi of ^{45}Ca (1.0 Ci/mmol). When glands were stimulated with 10 nM 5-HT, the rate of ^{45}Ca accumulation was increased. This increase was detected as early as 1 min after addition of ^{45}Ca, and was sustained for at least 40 min (Fig. 4*a*). When the effects of 5-HT and 10 mM cAMP upon ^{45}Ca uptake were compared 15 min after addition of ^{45}Ca, 5-HT caused approximately a 2-fold increase ($P < 0.001$), but the uptake of ^{45}Ca in the cAMP-treated glands was only 80% of that measured in the control gland (Fig. 4*b*).

Calcium Efflux. Either 5-HT or cAMP induced an increase in the rate of ^{45}Ca efflux from previously labeled cells (Fig. 5). However, the change in efflux pattern induced by the two agents differed. Addition of 5-HT caused an immediate and sharp rise in the rate of efflux with a peak in the first 5 min, after which the rate fell to nearly control levels in the next 10–15 min (Fig. 5*a*). Addition of cAMP caused a slower and less striking rise with a peak in 10 min, but with a more sustained increase in efflux (Fig. 5*c*). This difference in efflux pattern was abolished when the rate of efflux was measured in glands incubated in a medium containing 5 mM EGTA. Under these circumstances, both 10 nM 5-HT and

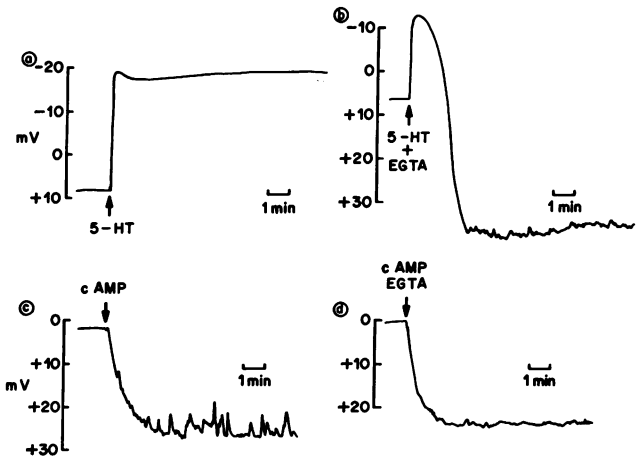

FIG. 3. The transepithelial potential (lumen referred to serosal medium) of the salivary gland when stimulated by 10 nM 5-HT in the presence (*a*) and absence (*b*) of calcium, or by 10 mM cAMP in the presence (*c*) or absence (*d*) of calcium. The measurements were made at 25° as described (25).

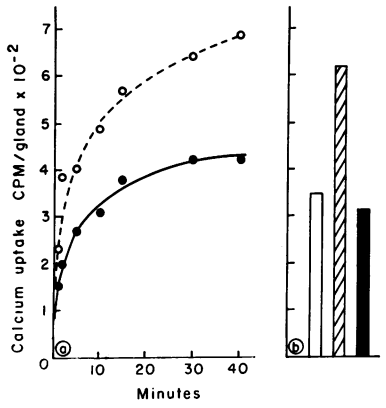

FIG. 4*a*. The uptake of ^{45}Ca by isolated fly salivary glands at 27° as a function of time in the absence (●—●) or presence of 10 nM 5-HT (O- - -O). 20 matched glands per tube were incubated at 27° in 500 μl of standard buffer (pH 7.7, ref. 22) containing 0.5 mM calcium and 10 μCi of ^{45}Ca. ^{45}Ca was added 5 min after addition of 5-HT or carrier to the respective tubes. At appropriate times after addition of ^{45}Ca, 5 ml of ice-cold buffer without labeled calcium were added and the tubes were placed on ice, then washed four times with the same buffer at 4°. After the final wash, all but 0.1 ml of buffer was removed. The scintillation fluid [1000 ml of toluene, 500 ml of Triton X-100 and 6g of butyl PBD (Ciba)] was added directly to these tubes; tube contents were then transferred to scintillation vials, and counted in a Packard Tricarb scintillation counter. Each of the values recorded is the mean of six experiments.

FIG. 4*b*. The uptake of ^{45}Ca by salivary glands, 15 min after the addition of ^{45}Ca; incubation was in media containing 0.5 mM Ca^{2+}: *control* (□), with 10 nM 5-HT (▣), or 10 mM cAMP (■). The values recorded are from four separate experiments. The mean ± standard error of the mean of the uptake by control glands was 340 ± 60 cpm/gland; of the 5-HT-treated glands 616 ± 110 cpm/gland; and of the AMP-treated glands, 308 ± 68 cpm/gland.

10 mM cAMP induced a similar change in the rate of ^{45}Ca efflux (Figs. 5*b* and *d*).

DISCUSSION AND CONCLUSION

Our results demonstrate that the same fundamental relationship exists between calcium and cAMP in the control of cellular activity in insect salivary gland tissue as has been previously found in a number of mammalian and amphibian tissues (7, 8, 17). The relationship between the action of calcium and cAMP within the cell is complex. Activation of secretion either by 5-HT or exogenous cAMP required calcium (Fig. 1). In both cases, the initial activation of calcium secretion seen in a calcium-free medium could be explained by the fact that both 5-HT and exogenous cAMP, through increasing intracellular cAMP, mobilized calcium from an intracellular pool (Fig. 5). As this mobilized calcium left the gland and was not replaced, because of the EGTA in the medium, secretion was not maintained (Fig. 1). In support of this argument is the fact that the time courses of secretory responses in calcium-free media (Fig. 1) and of ^{45}Ca efflux in similar media (Fig. 5) were similar.

The difference in the time course of change in secretory rate seen after addition of 5-HT, as compared to addition of cAMP, to glands incubated in EGTA (Fig. 1) can probably be accounted for by the ability of 5-HT to increase the influx of calcium that is present in the basement membrane

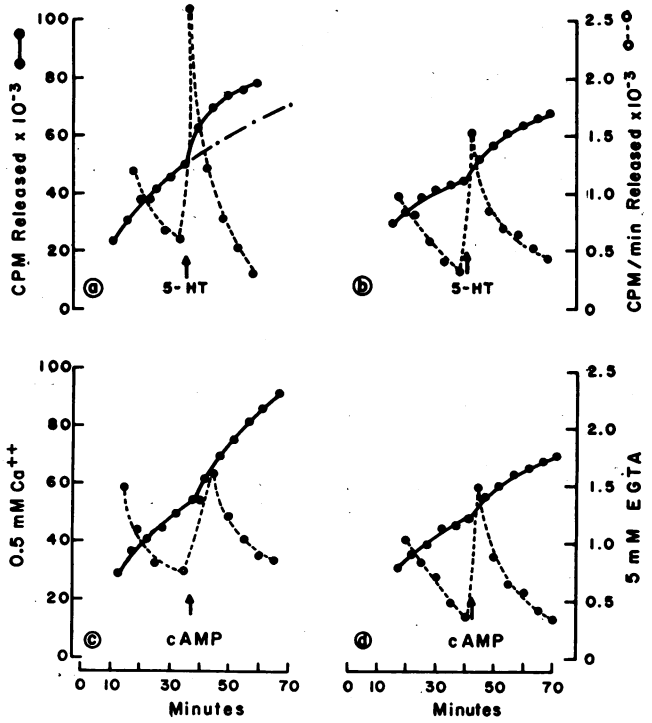

FIG. 5. The effect of 5-HT and cAMP on ^{45}Ca efflux from previously labeled salivary glands. Matched sets, containing 20 glands each, were incubated overnight at 4° in 0.5 ml of buffer containing 50 μCi of ^{45}Ca and 0.5 mM calcium (see legend to Fig. 1), warmed to 27°, incubated at that temperature for an additional 30 min, cooled to 4°, washed four times with buffer without ^{45}Ca, resuspended in 0.8 ml of the appropriate medium (see legend of Fig. 1), and transferred to a shaking water bath that was maintained at 27°. Beginning 15 min later, 50-μl samples of supernatant from each tube were removed at 5-min intervals and placed in 10 ml of toluene–Triton scintillation fluid. After six such samples had been removed, leaving 0.5 ml, 0.5 ml of the solution of the same medium only, or with 20 nM 5-HT, or 20 mM cAMP was added to the respective tubes (*arrows*). An additional six 100-μl samples were collected at 5-min intervals and each sample was added to 12 ml of scintillation fluid.

Efflux was measured in the presence of either 0.5 mM calcium (*a* and *c*) or 5 mM EGTA (*b* and *d*). The results have been expressed in two ways, either as the total cumulative counts released from the gland (●—●) or as the rate of efflux (○- - -○). The cumulative counts released from a control set of glands is shown in *a* (- · - · -). In each case shown (*a–d*), the results are from a single set of matched glands. Each experiment was repeated five times with essentially similar results.

region of the tissue for a short period during the change from a calcium-containing to a calcium-free solution. During this wash period, 5-HT stimulated an immediate influx of calcium from this temporary extracellular pool and subsequently mobilized calcium from an internal source by increasing the intracellular concentration of cAMP (Fig. 2). Since exogenous cAMP did not increase calcium influx (Fig. 4*b*), its action was confined to mobilizing calcium from the intracellular pool, thus leading to a slower rate of cell activation.

In addition to its role in association with the action of cAMP, calcium played a second role in association with the action of 5-HT. This is illustrated by the difference in the effect of 5-HT and cAMP upon transepithelial potential (Figs. 3*a* and *c*), calcium uptake (Fig. 4), calcium efflux in the presence of calcium (Figs. 5*a* and *c*), and the difference in rate of recovery of maximal rates of secretion after calcium was again added in cAMP-activated glands, compared to 5-HT-activated glands (Fig. 1). These differences indicate that 5-HT had an additional calcium-dependent effect upon membrane function in the salivary gland. The nature of this effect remains to be established, but on the basis of other studies (Prince and Berridge, unpublished), it may be a change in the chloride permeability of the luminal membrane. Regardless of the eventual explanation of these other effects of 5-HT, the present results emphasize that even though both 5-HT and exogenous cAMP induce the same physiological response, i.e., secretion, their modes of action are not identical.

The one aspect of cAMP action that remains to be examined in this insect tissue is the role of cAMP-dependent protein kinases in the secretory response. In all other respects, the present data are consistent with the previous model of cell activation (7). It appears that one of the fundamental effects of cAMP upon animal cell function is its effect upon cellular calcium metabolism, and that changes in the concentration of cytosolic calcium together with those of cAMP coordinate the intracellular events in many cells after their activation by specific extracellular messengers.

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