CHANGES IN CALCIUM TRANSPORT ACROSS CALLIPHORA SALIVARY GLANDS INDUCED BY 5-HYDROXYTRYPTAMINE AND CYCLIC NUCLEOTIDES

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SUMMARY

The efflux of $^{45}$Ca from prelabelled salivary glands was studied under a variety of conditions. 5-Hydroxytryptamine (5-HT) or cyclic AMP caused a large release of label most of which entered the saliva. The ionophore A23187 caused a similar release of calcium but EGTA had no effect.

The presence of an active calcium pump on the luminal surface was investigated further by studying calcium transport across the gland. This transport of calcium, which provides a measure of calcium entry into the cell, was very sensitive to 5-HT concentration but little affected by cyclic AMP. The small cyclic AMP-dependent transport was depressed by 8-bromo cyclic GMP with a parallel fall in the rate of fluid secretion.

These studies provide more direct evidence that 5-HT acts to stimulate the entry of external calcium in addition to mobilizing internal calcium.

INTRODUCTION

Calcium plays an essential role in stimulus-secretion coupling in a wide range of secretory cells (Rubin, 1970; Berridge, 1975). While its function in triggering the release of preformed granules or vesicles by exocytosis is well established, there is less information on its possible role as a second messenger in fluid secretion. In mammalian salivary glands, there is indirect evidence linking calcium entry with the efflux of potassium which seems to accompany the onset of fluid secretion in response to $\alpha$-adrenergic and cholinergic agents (Schramm & Selinger, 1975).

In the case of the fly salivary gland, tracer ($^{45}$Ca) studies have provided indirect evidence that calcium is an important second messenger in initiating the increased flow of saliva induced by 5-hydroxytryptamine (5-HT) (Prince, Berridge & Rasmussen, 1972). In order to gather further evidence that calcium is important in cell activation, more direct studies were carried out again using $^{45}$Ca to trace the movement of calcium. In the first series of experiments, the efflux of $^{45}$Ca from prelabelled glands either into the bathing medium or into the saliva was studied under a variety of conditions. Most of the calcium was found in the saliva suggesting that calcium

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Salivary gland

Fig. 1. Diagrammatic representation of the techniques used for measuring either calcium efflux or calcium transport. For the efflux studies the gland was preincubated in $^{45}$Ca so as to label the intracellular pools of calcium (a, I). The $^{45}$Ca was washed away from the medium bathing the closed end of the gland and the efflux of $^{45}$Ca either to the medium (a) or to the saliva (b) was then measured at set intervals. For the transport studies, $^{46}$Ca was added to the medium (b, II) and its rate of appearance in the saliva (c) was measured.

was preferentially extruded from the cell across the lumenal membrane. Under normal conditions, therefore, most of the calcium which enters the cell across the basal membrane will be transported into the lumen thus producing a net transport of calcium across the gland. Since the entry of calcium across the basal membrane is the rate-limiting step, a reasonable estimate of the permeability of this membrane can be obtained simply by measuring the net transport of calcium across the gland. The second series of experiments are concerned with the effects of 5-HT and cyclic nucleotides on this net transport of calcium across the gland.

**METHODS**

The salivary glands of adult *Calliphora* were used throughout this study. The technique for setting up salivary glands *in vitro* was described previously (Berridge & Patel, 1968). The artificial saline had the following composition (mM): Na 155; K 20; Ca 2; Mg 2; Tris 10; Cl 156; phosphate 2; malate 2.7; glutamate 2.7. Where amylase activity was assessed on the saliva alone, the medium also contained 10 mM-glucose. Glucose was omitted when the medium was assayed for amylase. Phenol red was added to keep a continuous check on the pH which was maintained between 7.2 and 7.4. The ionophore A23187 was made up in a calcium-free saline containing 5 mM EGTA as described previously (Prince, Rasmussen & Berridge, 1973).

**Calcium flux and transport measurements**

In order to study the efflux of calcium, salivary glands were prelabelled with radiocalcium using two separate techniques. In the initial experiments six salivary glands were set up under liquid paraffin and the drop of saline surrounding the
Changes in calcium transport

Fig. 2. The effect of $1 \times 10^{-4}$ M 5-HT on (a) the rate of fluid secretion (b) calcium efflux into the saliva or medium (c) amylase secretion into saliva or medium. One unit of amylase is defined as that amount of enzyme liberating 1 µg of reducing equivalents in 5 min at 37 °C.

closed end of the gland was replaced with saline containing $^{46}$Ca ($3 \times 10^7$ cpm/ml). The glands were left for 16 h at 4 °C after which the labelling solution was removed by repeated washing. In practice, five washings were enough to reduce the $^{46}$Ca content of the bathing medium to a sufficiently low level to begin the efflux measurements. At this stage, the glands were set up in the usual manner and the rate at which $^{46}$Ca (I in Fig. 1) was released either to the bathing medium (Fig. 1a) or to the saliva (Fig. 1b) was measured. The medium and saliva samples collected at 2 or 5 min intervals were diluted in 200 µl of chilled saline and aliquots were taken for measurement of $^{46}$Ca and amylase according to Bernfeld (1955). In subsequent experiments it was found that a 15 min incubation with $^{46}$Ca ($2.3 \times 10^7$ cpm/ml) at room temperature was sufficient to label the gland for efflux measurements. At the
conclusion of some experiments, the glands were digested with Hyamine (Packard Radiochemicals, LaGrange, Ill., U.S.A.) and the residual radioactivity assessed.

For the transport studies $^{45}\text{Ca}$ was added to the outside medium (II in Fig. 1) and the rate at which calcium appeared in the saliva (Fig. 1c) was measured.

All the samples containing $^{45}\text{Ca}$ were added to Bray's solution and counted in a Packard Tricarb Scintillation counter.

**RESULTS**

**Calcium efflux and amylase secretion**

5-HT produced a very large increase in the efflux of $^{45}\text{Ca}$ into the saliva whereas little calcium entered the bathing medium (Fig. 2). It seems unlikely that this appearance of $^{45}\text{Ca}$ in the saliva is associated with the release of amylase because there was a clear temporal separation in the time course of these two events (Fig. 2). During the first treatment with 5-HT the onset of amylase secretion developed slowly whereas the efflux of calcium into the saliva was maximal within the first 5 min. Furthermore, during the second treatment with 5-HT there was a large release of amylase but the calcium efflux was reduced. The decline in $^{45}\text{Ca}$ could not be ascribed to depletion of the total calcium sequestered in the various depots in the gland, since in no instance were the cells and associated membranes emptied of more than 60% of the total $^{45}\text{Ca}$ taken up. Analysis of the digested glands at the conclusion of each experiment revealed that from one half to one third of the residual activity remained associated with the tissue regardless of the procedure used for loading and washing of the tissue.

The divalent ionophore A23187 ($2 \times 10^{-7} \text{M}$) also produced a large increase in calcium efflux but, in this case, calcium appeared in both the medium and the saliva (data not shown). The ionophore had little effect on amylase release which was already somewhat elevated prior to the addition of the ionophore. The calcium-free saline used in these experiments may have contributed to the enhanced basal level of amylase secretion. These experiments again demonstrate the dissociation between calcium efflux and amylase release, because A23187 induced a large change in calcium efflux with little or no change in the rate of amylase secretion.

The observation that much of the $^{45}\text{Ca}$ which leaves the gland enters the saliva greatly complicates the analysis of these efflux measurements. There are also practical difficulties in studying the efflux of $^{45}\text{Ca}$ into the lumen because, unless the lumen can be perfused at a constant rate, the rate of efflux will be very dependent on the rate of fluid secretion. For example, when the glands are at rest there will be a slow rate of secretion and $^{45}\text{Ca}$ will accumulate in the lumen thus making it difficult to establish the basal rate of calcium efflux. In the light of such difficulties we have concentrated on the smaller efflux of calcium into the bathing medium.

The rapid labelling technique was used to study the calcium efflux into the bathing medium under a variety of treatments. The small increase in calcium efflux into the bathing medium shown in Fig. 2 was found to be a consistent feature of the action of 5-HT (Fig. 3a). A similar increase in calcium efflux was observed during the action of $1 \times 10^{-2} \text{M}$ cyclic AMP which also produced an increase in the rate of fluid secretion (Fig. 3b). The subsequent addition of 5-HT caused a further
Changes in calcium transport

Fig. 3. The effect of different treatments on the rate of fluid secretion (nl/min, upper curves) and calcium efflux into the medium (cpm/gland/2 min, lower curves). (a) $1 \times 10^{-5}$ M 5-HT; (b) $1 \times 10^{-4}$ M cyclic AMP followed by $1 \times 10^{-4}$ M 5-HT; (c) $1 \times 10^{-4}$ M 5-HT administered during treatment with 100 mM-potassium; (d) the effect of $1 \times 10^{-4}$ M 5-HT after prior treatment in a calcium-free saline (5 mM EGTA).

small increase in fluid secretion but there was no change in calcium efflux. The small and slow increase in fluid secretion seen during the action of 100 mM-potassium caused a corresponding small increase in calcium efflux which was considerably increased during the subsequent action of 5-HT. The calcium which is being mobilized by these different stimulants is not accessible to EGTA which had no effect on the rate of calcium efflux (Fig. 3d). These experiments suggest that $^{46}$Ca is entering some intracellular pool from which it is released during cell activation. However, before we can accept such an interpretation it is important to exclude the possibility that the calcium which appears in the medium is not leaking out from
the large amount of calcium which is being released into the lumen. If the junctions between the cells were leaky to calcium, much of the $^{45}\text{Ca}$ which appears in the medium may have come from the lumen. In order to find out if there is an appreciable paracellular calcium shunt, we studied the transport of calcium from the medium into the saliva (Fig. 1$c$).

**Transepithelial calcium transport**

When $^{45}\text{Ca}$ was added to nonradioactive salivary glands which were secreting rapidly in the presence of $1 \times 10^{-7} \text{M} \ 5\text{-HT}$, $^{45}\text{Ca}$ appeared in the saliva very rapidly (Fig. 4). The saliva/medium ratio was surprisingly high (0.38) and indicated that large amounts of calcium were moving across the gland. The next experiment was designed to determine the route of this calcium movement. If calcium was moving via the paracellular shunt, it should be possible to decrease the $^{45}\text{Ca}$ concentration in the saliva simply by speeding up the rate of secretion thus allowing less time for equilibrium between medium and saliva. This prediction was not substantiated because when the rate of secretion was increased by raising the concentration of $5\text{-HT}$, the concentration of $^{45}\text{Ca}$ in the saliva did not decrease but instead it increased. Further increases in the $5\text{-HT}$ concentration caused no further changes in the rate of secretion but there were large increases in the $^{45}\text{Ca}$ concentration of the saliva. These experiments argue against the existence of a paracellular shunt and suggested that $^{45}\text{Ca}$ was moving through the cells.

The relationship between $5\text{-HT}$ concentration, fluid secretion and calcium transport is illustrated in Fig. 5. As the concentration of $5\text{-HT}$ was increased above $10^{-6} \text{M}$, there was a sudden increase in fluid secretion which reached a maximum at $10^{-6} \text{M}$ as noted previously (Berridge, 1970; Berridge & Prince, 1972). Calcium transport showed a similar sensitivity to $5\text{-HT}$ as fluid secretion but the increase in $^{45}\text{Ca}$ transport continued to increase linearly up to a maximum at $10^{-6} \text{M} \ 5\text{-HT}$ (Fig. 5).
Changes in calcium transport

This ability of 5-HT to enhance calcium movement across the gland was not shared by cyclic AMP (Fig. 6). During the action of cyclic AMP there was a small and gradual appearance of $^{45}$Ca in the saliva which was enormously increased when the cyclic AMP stimulation was briefly interrupted with a short treatment with $5 \times 10^{-8} \text{M}$ 5-HT. A characteristic feature of the effect of cyclic AMP on $^{45}$Ca transport is that it developed much more slowly than that observed during the action of 5-HT. If very small amounts of $^{45}$Ca were entering the gland during cyclic AMP stimulation, some of this calcium will be mopped up by the intracellular reservoirs and will not be available to enter the lumen. As more of the reservoirs become labelled, more $^{45}$Ca will then be available for transport into the lumen thus accounting for the gradual accumulation of $^{46}$Ca in the saliva. In order to test this possibility, two groups of salivary glands were stimulated with $10^{-2} \text{M}$ cyclic AMP. $^{45}$Ca was
Fig. 7. The rate of appearance of $^{44}$Ca in the saliva of two groups of salivary glands stimulated throughout the experiment with $10^{-4}$ M cyclic AMP. $^{44}$Ca (3400 cpm/µl) was added to the medium surrounding the group A glands for two separate periods (solid bars) whereas the group B received $^{44}$Ca during the second period only. The saliva from six glands was pooled in order to provide sufficient counts, the results are expressed as cpm/gland for each 4 min collection interval.

Fig. 8. The ability of 8-bromo-cyclic GMP ($10^{-4}$ M) to depress both the rate of fluid secretion (a) as well as the rate of calcium transport (b).
Changes in calcium transport

Fig. 9. A summary of the mechanisms thought to be responsible for regulating the free intracellular level of calcium in Calliphora salivary gland. 5-HT seems to promote an influx of external calcium (a) much of which is transported into the lumen (e) although some calcium also seems to leave the cell via the basal surface (d) or is sequestered into intracellular reservoirs (c). 5-HT also increases the intracellular concentration of cyclic AMP which may act to release calcium from intracellular reservoirs (b). The calcium released from these reservoirs may leave the cell via the two separate extrusion mechanisms (d and e) to account for the increased efflux of $^{46}$Ca from prelabelled cells during the action of either 5-HT (Figs. 2 and 3) or cyclic AMP (Fig. 3). The calcium transport studies seem to argue against an appreciable paracellular flow of calcium (f).

added to the first group (A) at 8 min and there was the usual gradual appearance of $^{46}$Ca in the saliva (Fig. 7). At 28 min, the $^{46}$Ca was removed and the amount of $^{46}$Ca appearing in the saliva fell rapidly to a low level but when $^{46}$Ca was readmitted at 34 min the calcium content of the saliva increased much more rapidly than it had previously. In contrast, when $^{46}$Ca was added to the control glands (Group B) at 34 min, there was the same slow rate of $^{46}$Ca transport similar to that seen in the Group A glands during their first treatment.

The 8-bromo derivative of cyclic GMP was capable of markedly depressing the slow rate of calcium transport observed during stimulation of the glands with cyclic AMP (Fig. 8). This suppression of calcium transport was associated with a decrease in the rate of fluid secretion. When glands were stimulated with 5-HT, 8-bromo cyclic GMP had no effect on the high rate of calcium transport.
The role of calcium in the action of 5-HT on *Calliphora* salivary glands was first established by studying both the efflux and the uptake of $^{45}$Ca (Prince *et al.*, 1972). 5-HT, but not cyclic AMP, was able to increase the uptake of calcium whereas both agents were capable of enhancing the efflux of calcium from prelabelled glands. These observations were incorporated into a model which is summarized in figure 9. One proposed action of 5-HT was to increase the influx of calcium (Fig. 9a) which thus accounted for the increased uptake of $^{45}$Ca. Another action of 5-HT was to increase the intracellular level of cyclic AMP (Prince *et al.* 1972) which was postulated to bring about a release of calcium from some intracellular reservoir (Fig. 9b). When cells are labelled with $^{45}$Ca, most of the label accumulates in intracellular reservoirs and the subsequent rate of efflux is determined mainly by the rate at which this calcium leaves the reservoirs (Fig. 9b) to become available to the extrusion mechanisms on the surface membrane (Fig. 9d, e). The proposal that cyclic AMP can release calcium from these stores would thus explain the enhanced efflux of $^{45}$Ca seen with either 5-HT or cyclic AMP (Prince *et al.* 1972). In this earlier study no attempt was made to separate efflux into the medium (Fig. 9d) from efflux into the saliva (Fig. 9e).

An important feature to emerge from the experiment shown in figure 2, was that most of the efflux occurred into the saliva, only a small amount of $^{45}$Ca was released into the medium. Most of the efflux recorded previously was clearly coming from the lumen which raises the possibility that it may have been associated with the release of amylase and may not have been a reliable indicator that internal calcium was being mobilized during cell activation. It is known from studies on mammalian salivary glands that large amounts of calcium are accumulated in the amylase granules and are released during secretion (Wallach & Schramm, 1971). The salivary glands of *Calliphora* also contain amylase granules which are released into the lumen during the action of 5-HT or cyclic AMP (Hansen Bay, 1976). However, there are several reasons for excluding the possibility that the $^{45}$Ca which was released into the lumen of *Calliphora* salivary glands was associated with the extrusion of amylase granules. Firstly, the calcium which is packaged into amylase granules is thought to exchange very slowly so it is unlikely that a significant proportion of the $^{45}$Ca which entered the cell will have been incorporated into the granules especially in those experiments where the glands were labelled for a short period. Secondly, when $^{45}$Ca efflux and amylase release were compared there appeared to be a temporal separation of these two events (Fig. 2). Thirdly, during the action of A23187 there was a very large increase in calcium efflux with little change in amylase secretion. The large increase in calcium efflux into the lumen was probably the result of a calcium extrusion mechanism (Fig. 9e) and was apparently not associated with amylase release. Further evidence for the existence of a calcium pump on the apical membrane has emerged from the transport studies as discussed later. We conclude, therefore, that during the action of 5-HT there is an increased mobilization of internal calcium and much of this leaves the cell via the luminal surface (Fig. 9e) whereas a smaller proportion is transported across the basal surface (Fig. 9d) to account for the increased efflux of $^{45}$Ca into the medium (Fig. 3). The calcium transport studies showed that there
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was little leakage of calcium through the cell junctions (Fig. 9f) because very little calcium entered the saliva when glands were stimulated with cyclic AMP or with low doses of 5-HT. Therefore, the appearance of $^{46}$Ca in the medium during the course of an efflux study is probably not a consequence of $^{46}$Ca leaking back into the medium from the saliva, but is most likely a true reflection of calcium extrusion from the cell across the basal membrane (Fig. 9d). The ability of both 5-HT and cyclic AMP to increase this efflux of $^{46}$Ca seems to substantiate the idea that there is a cyclic AMP-dependent mobilization of internal calcium (Fig. 9b) as was previously postulated (Prince et al. 1972).

Although there was little evidence for a paracellular calcium shunt, there was considerable evidence for a 5-HT-dependent flow of calcium through the cell. The fact that the transport of calcium was related to 5-HT concentration (Fig. 5) is certainly consistent with the model outlined earlier that the entry of calcium (Fig. 9a) is regulated by 5-HT. Once calcium enters the cell it becomes susceptible to the various uptake (Fig. 9c) or extrusion mechanisms (Fig. 9d and e). It was argued earlier, on the basis of the calcium efflux measurements (Fig. 2) that the pump on the lumenal surface (Fig. 9e) is more active than the pump on the basal surface (Fig. 9d) which would explain why large amounts of calcium were transported into the saliva (Fig. 2). These observations on calcium transport are thus consistent with previous studies which showed that 5-HT was able to stimulate $^{46}$Ca uptake whereas cyclic AMP had no effect (Prince et al. 1972).

Calcium entry seems to be linearly related to the concentration of 5-HT over a much wider range than that observed for the activation of fluid secretion. 5-HT continued to increase the entry of calcium at concentrations which were far in excess of those necessary to bring about a maximal stimulation of fluid secretion (Fig. 5). Ozato, Huang & Ebert (1977) have also observed that concanavalin A continues to stimulate the uptake of calcium into murine thymocytes at concentrations which are far in excess of those necessary for a maximal mitogenic effect (Hesketh et al. 1977). A similar phenomenon has been observed in other cells with respect to cyclic AMP. In the liver, glucagon elevates the level of cyclic AMP far in excess of that necessary to bring about a maximal activation of glycogen metabolism (Exton, Robison, Sutherland & Park, 1971). This excessive production of second messengers probably reflects the existence of spare receptors (Levitzki, 1976). Most cells have a large population of receptors of which only 0.01 to 1 % need to be occupied in order to activate the cells maximally (Levitzki, 1976). The redundant synthesis of cyclic AMP or entry of calcium at high hormone concentrations may reflect an increase in the number of hormone-receptor interactions as more and more of these spare receptors are brought into play.

The possible role of cyclic GMP in the control of secretion by Calliphora salivary glands has not been studied in detail. Cyclic GMP was not able to stimulate fluid secretion when applied to the gland (Berridge, 1973). While it clearly lacks any stimulatory effect, cyclic GMP may exert an inhibitory effect by suppressing the entry of calcium. Although the 8-bromo derivative of cyclic GMP was able to reduce the low rate of calcium entry observed during stimulation with cyclic AMP (Fig. 8), it had no effect on the high rate of calcium entry found during stimulation with 5-HT. If cyclic GMP has an effect on calcium permeability, the
inhibitory effect may be apparent only at low rates of calcium entry. A similar inhibitory action of cyclic GMP on calcium entry has been put forward by Schultz, Schultz & Schultz (1977) in order to explain the ability of this 8-bromo derivative of cyclic GMP to relax smooth muscle.

REFERENCES


