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# STUDIES ON THE MECHANISM OF FLUID SECRETION BY ISOLATED SALIVARY GLANDS OF *CALLIPHORA*

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SUMMARY

1. Potassium is the major cation in the secretion of the salivary glands of *Calliphora* and is necessary for full secretory rates.

2. Other ions (rubidium and sodium) can support secretion in the absence of potassium.

3. During stimulation with 5-HT a Nernst plot of the basal membrane potential has a slope of 53 mV for a tenfold change in external potassium concentration and the slope at rest deviates from this over the range 1–20 mM external potassium.

4. Hyperpolarization of the basal membrane by 5-HT is abolished if the chloride in the bathing medium is replaced by isethionate.

5. The diuretic agent amiloride inhibits fluid secretion by a mechanism which may include a reduction in calcium entry in addition to its recognized effect on sodium permeability.

6. A model is proposed in which fluid secretion is driven by the active transport of potassium across the apical membrane with chloride following passively.

Previous studies on isolated salivary glands of *Calliphora* have concentrated on the role of cyclic AMP and calcium in mediating the action of 5-hydroxytryptamine (5-HT) (Berridge, 1970; Berridge & Prince, 1972*a*, *b*; Prince & Berridge, 1972, 1973; Prince, Berridge & Rasmussen, 1972). Electrophysiological studies suggested that cyclic AMP stimulated a cation pump whereas calcium increased anion permeability. The role of calcium in altering membrane permeability has been confirmed by measuring the resistance of both the apical and basal membranes during the action of 5-HT (Berridge, Lindley & Prince, 1975*a*). These resistance measurements have been used to develop a model which adequately accounts for all the potential changes normally observed during the action of 5-HT. The basis of this model depends on having an active pump on the apical membrane which transports potassium from the cell into the lumen. Chloride follows passively. Potassium and chloride transported

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from the cell into the lumen is replaced by a passive influx of these ions across the basal plasma membrane into the cell.

The experiments described in this paper were performed to investigate the properties of the ion transporting system in this tissue. This entails studying both the pump on the apical membrane and the permeability properties of the basal membrane which can severely alter pump activity by regulating the entry of ions into the cellular compartment.

## METHODS

The methods used in this paper were the same as those described previously and involved the measurement of secretory rates (Berridge & Patel, 1968), transepithelial and intracellular potential (Berridge & Prince, 1972*b*; Prince & Berridge, 1972), transepithelial resistance (Berridge *et al.* 1975*a*) and cyclic AMP (Prince *et al.* 1972). Sodium and potassium concentration in the saliva and bathing medium were measured by means of a Beckman flame-photometer.

The 'normal saline' used in the electrical experiments was the same as that used before and 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, glucose 10. Phenol red (< 0·01 mM) was routinely added to keep a continuous check on the pH which was maintained between 7·2 and 7·4. In the experiments described in this paper a variety of other salines were used. When the potassium concentration was varied the sodium concentration was also varied so as to maintain the sum of these two cations together at 175 mM. In sodium or potassium-free saline ionic balance was maintained with Tris-buffer. In chloride-free experiments sodium chloride was replaced by isethionate and other chlorides by the corresponding sulphates.

When the effects of individual cations were tested on the rate of fluid secretion, each cation was presented at a concentration of 150 mM and ionic balance was maintained with Tris. When the potassium concentration was varied the ionic balance was maintained with either sodium or Tris.

## RESULTS

### *The effect of different cations on the composition and rate of saliva secretion*

A striking feature of the isolated salivary glands of *Calliphora* is their ability to secrete fluid when supplied with different univalent cations (Table 1). The rates of secretion shown in Table 1 were obtained 10 min after stimulating the glands with 5-HT. The glands display a preference for potassium and rubidium but high rates of secretion were observed in the pure sodium medium.

Since the normal medium contains both sodium and potassium it was of interest to study the effect of varying the ratio of these two cations, In pure sodium the rate was approximately 62·5 % of that observed in normal saline (Fig. 1*a*). Replacement of sodium with small amounts of potassium increased the rate of secretion so that normal rates were recorded when the external medium contained 5 mM potassium. Further increases in potassium had no additional effect. In the complete absence of potassium, sodium was the major cation in the saliva, but with the addition of small quantities of potassium (over the range 0–10 mM) the predominant cation in the saliva changed over

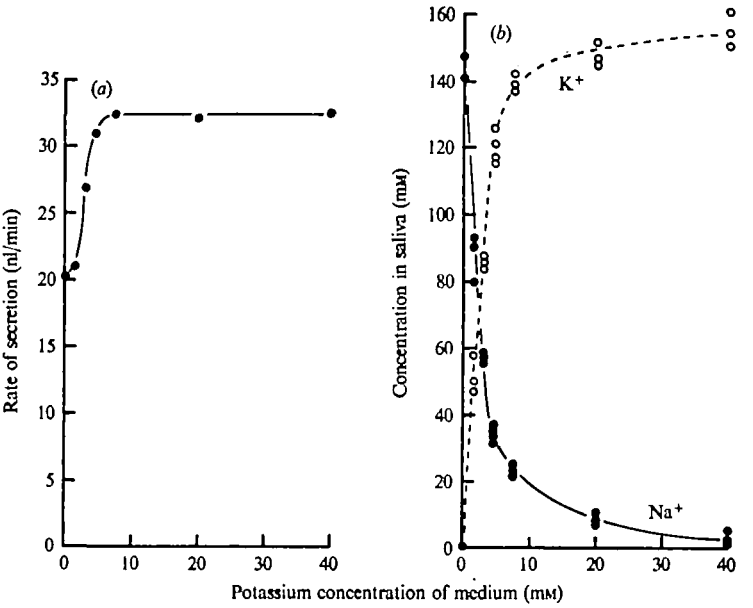


Fig. 1. The effect of potassium on the secretion and composition of saliva secreted during stimulation of isolated salivary glands with $10^{-8}$ M 5-HT. The potassium concentration was varied from 0–40 mM and the ionic balance was maintained with sodium. (*a*) Rate of fluid secretion; (*b*) the sodium (●) and potassium (○) concentration of the saliva secreted during (*a*).

Table 1. *Effect of different monovalent cations on the rate of fluid secretion by isolated salivary glands*

| Cation | Rate of secretion (nl/min) |
|---|---|
| $K^+$ | 38·5 |
| $Rb^+$ | 38·5 |
| $Na^+$ | 20·0 |
| $Cs^+$ | 7·0 |
| $Li^+$ | 5·2 |
| $Tris^+$ | 2·4 |

rapidly from sodium to potassium (Fig. 1 *b*). With further rises in the external potassium concentration the sodium level of the saliva declined more gradually (Fig. 1 *b*). These results show that the glands have a very marked preference for potassium since they selectively secreted this cation despite the large excess of sodium available in the bathing medium.

In the next series of experiments these two cations were studied independently and, in each case, the cation balance was maintained with Tris. In a Tris medium the rate of secretion was very low (Table 1) but increased linearly over the range 0–10 mM external potassium to normal secretory rates (Fig. 2). Thus, even in the absence of sodium, the glands were capable of maintaining high rates of secretion at very low external potassium concentrations. When Tris was replaced with sodium a linear relationship between rate of secretion and sodium concentration was observed which

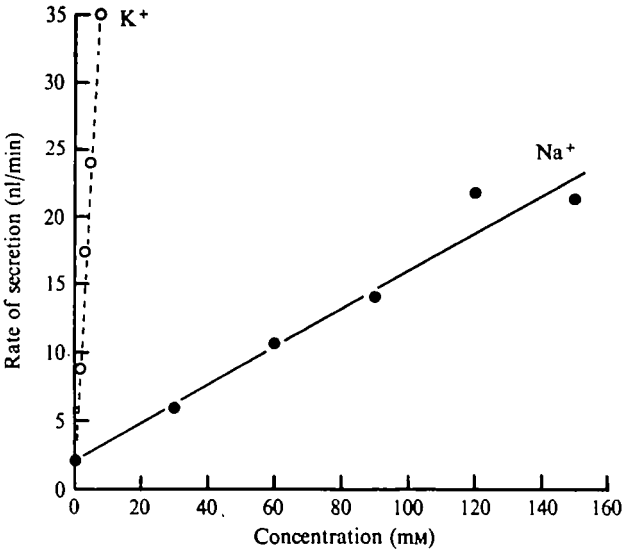


Fig. 2. The rate of secretion of salivary glands stimulated with $10^{-6}$ M 5-HT in salines containing potassium (○) or sodium (●). The cation balance was maintained in these experiments by Tris.

extended over a wide concentration range (0–150 mM). At the highest concentration the rate was only 60 % of the rates seen in low potassium media (Fig. 2). These results clearly indicate that sodium is less effective than potassium in supporting fluid secretion.

*The effect of variations in the external potassium concentration on basal membrane potential*

The effect of varying the external potassium concentration on the potential across the basal membrane has been studied both at rest and during stimulation with 5-HT (Fig. 3*a*). In another series of experiments the effect of replacing chloride with isethionate was studied over a limited range of external potassium concentrations (Fig. 3*b*). During stimulation with 5-HT in normal saline the potential measurements fit a straight line with a slope of 53 mV for a tenfold change in the external potassium concentration. At rest the situation is more complicated as the points lie on a line with a slope of 53 mV at high potassium concentrations (above 20 mM) but on a line with a slope of 44 mV at low potassium concentrations (below 20 mM) (Fig. 3*a*). There was some indication that there were changes in intracellular potassium concentration during changes in external potassium because the potential responses to potassium changes were asymmetric. The half times ($t_{\frac{1}{2}}$) for changing from high to low potassium were always slower than changes from low to high potassium, i.e. going from 20 to 3 mM $t_{\frac{1}{2}}$ equalled $5{\cdot}2 \pm 1{\cdot}35$ s ($n = 7$) and from 3 mM to 20 mM $2{\cdot}42 \pm 0{\cdot}6$ s whilst from 20 to 1·5 mM $t_{\frac{1}{2}}$ equalled $9{\cdot}6 \pm 2{\cdot}7$ and from 1·5 to 20 mM $3{\cdot}16 \pm 0{\cdot}6$. These differences were significant at the 0·01 level.

When the chloride in the bathing medium was replaced by isethionate the slope of the line obtained was 49 mV for a tenfold potassium change and thus lay between the stimulated and unstimulated points obtained in normal saline (Fig. 3*b*). Unlike

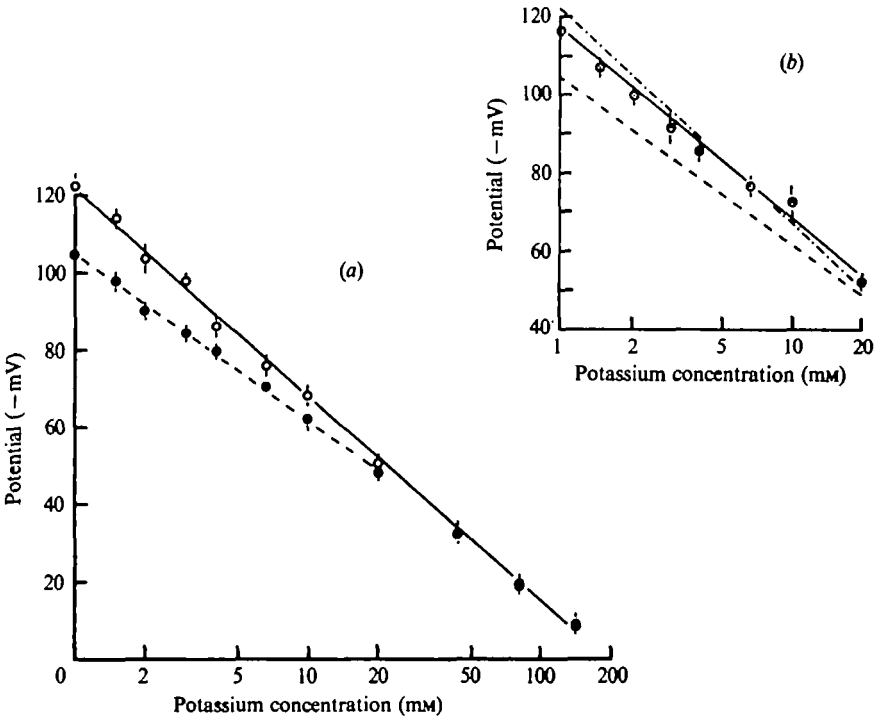


Fig. 3. (*a*) The potential (mV) of the basal membrane at different concentrations of potassium (mM) in the bathing medium at rest (●) and during stimulation with $10^{-6}$ M 5-HT (○). The lines have slopes of 53 mV (continuous line) and 44 mV (dashed line) for a tenfold change in the potassium concentration of the bathing medium. Above 20 mM the two points were superimposed. (*b*) The potential of the basal membrane during stimulation with 5-HT at different concentrations of potassium in the bathing medium when chloride is replaced by isethionate (⊙). The potential of cells at rest in the same medium are omitted for clarity but fall on the same line with a slope of 49 mV for a tenfold change in the potassium concentration (continuous line). The other lines are those from Fig. 3*a* for comparison. In *a* and *b* each point represents the average of eight cells ($\pm 2x$ S.E.M.). The lines were fitted by linear regression analysis.

the situation in the normal chloride-containing medium there was no change in the slope of this line after stimulation with 5-HT. After the addition of 5-HT to glands in a chloride-containing saline there was a small, rapid hyperpolarization of the basal membrane which was followed by a larger, slow hyperpolarization (Fig. 4*a* and Prince & Berridge, 1972). In the absence of chloride, however, there was very little change in the basal membrane potential after the addition of 5-HT (Fig. 4*b*). It should be noticed here that the size of responses recorded in normal chloride-containing saline were increased when the potassium concentration was lowered.

*The effect of potassium-free media on potential responses*

Since sodium alone was capable of supporting fluid secretion it was of interest to compare the potential responses of glands in these potassium-free media with those in the control medium (Fig. 5). During short pulses of 5-HT the transepithelial potential displayed typical biphasic responses as previously described (Berridge & Prince, 1971, 1972*a*). The basal plasma membrane responded with typical small

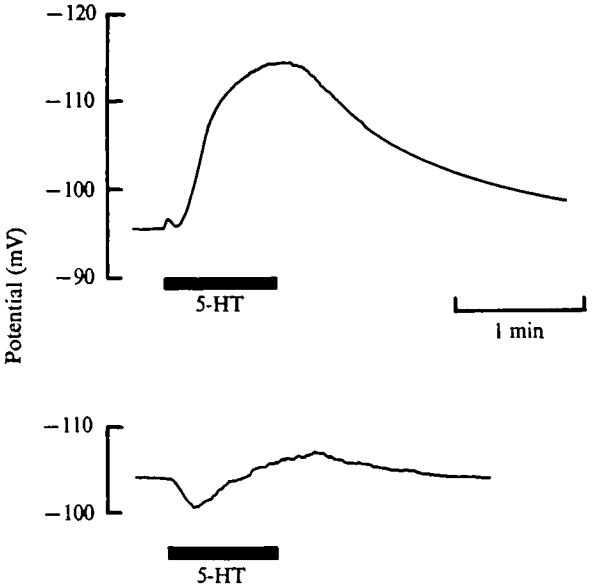


Fig. 4. Potential responses of the basal membrane to 5-HT applied for the duration of the solid bars in the presence of chloride (top trace) and when chloride was replaced by isethionate (bottom trace). In both records the potassium concentration of the bathing medium was 2 mM.

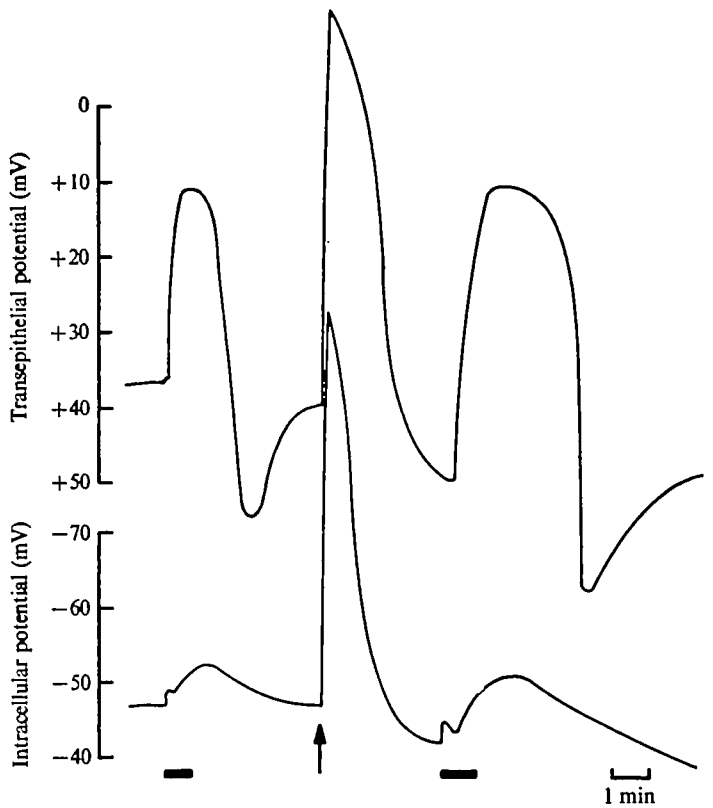


Fig. 5. Transepithelial (top trace) and intracellular (bottom trace) potential records showing responses to 5-HT (applied for the duration of the solid bars) in normal saline containing 20 mM potassium and after perfusion with a potassium-free saline (salines changed at arrow).

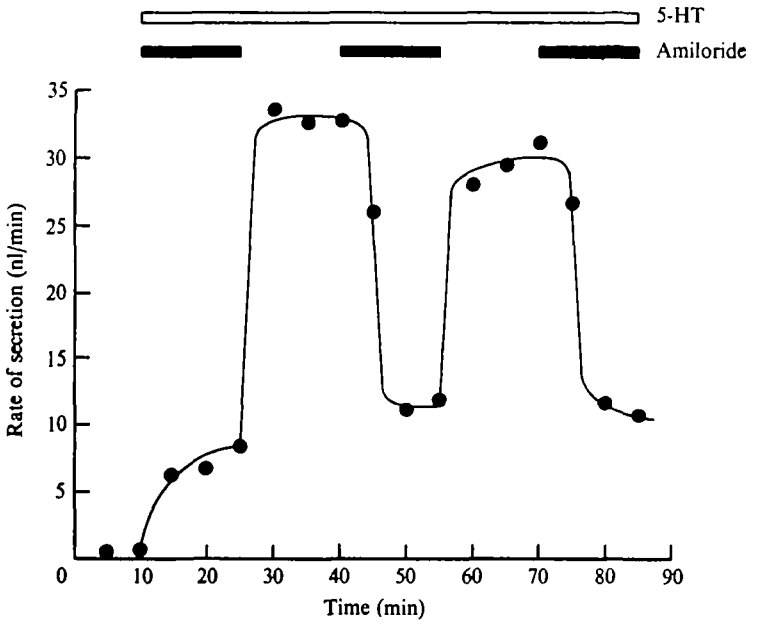


Fig. 6. The inhibitory effect of $2 \times 10^{-4}$ M amiloride (solid bars) on fluid secretion by salivary glands in normal saline during stimulation with $1 \times 10^{-6}$ M 5-HT (open bar).

hyperpolarizations (Prince & Berridge, 1972). When the control medium was replaced with a potassium-free saline, where sodium replaced potassium, there was an immediate and large hyperpolarization of the basal membrane. After 1 min, however, the potential gradually drifted back to the resting level. This change in the basal membrane potential was reflected in the transepithelial potential which went negative before returning to its original level. Having adapted to this new external environment the glands responded to 5-HT in qualitatively the same manner as in control medium. However, the hyperpolarization across the basal membrane was exaggerated. The transepithelial potential change was slower than normal and the response lasted longer (in Fig. 5 the response is approximately 50 % longer). If the glands were returned to control medium they responded normally to 5-HT.

*The effect of various drugs known to interfere with ion transport*

Salivary glands were insensitive to ouabain (when treated for up to 30 min with $10^{-4}$ M). Ethacrynic acid, which is a less specific inhibitor of membrane-bound ATPases, inhibited fluid secretion and abolished the positive phase of the transepithelial potential which has been attributed to pump activity (Berridge & Prince, 1972*b*). However, inhibition was apparent only at relatively high concentrations ($1 \times 10^{-4}$ M) and was irreversible.

The diuretic agent amiloride, which is supposed to inhibit the passive movement of sodium, was capable of reversibly inhibiting fluid secretion (Fig. 6). The effect was apparently specific for sodium because if glands were treated with excess potassium there was no inhibition (Fig. 7*a*). In the high potassium salines the rate of secretion increased spontaneously without the addition of 5-HT. This phenomenon is described

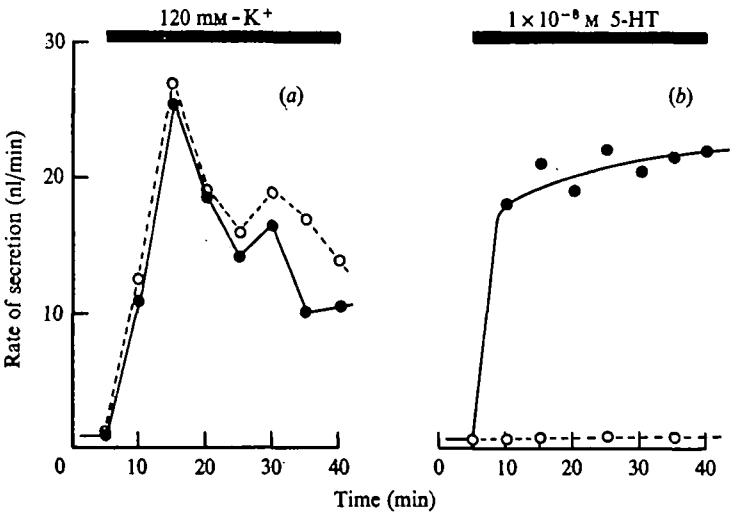


Fig. 7. The inhibitory effect of $2 \times 10^{-4}$ M amiloride on fluid secretion in salines of differing ionic composition. (*a*) Salivary glands were treated with 120 mM potassium which is capable of stimulating secretion in the absence of 5-HT. (*b*) Salivary glands stimulated with $1 \times 10^{-6}$ M 5-HT in a potassium-free saline containing 150 mM sodium. ●, control response; ○, response in presence of $2 \times 10^{-4}$ M amiloride.

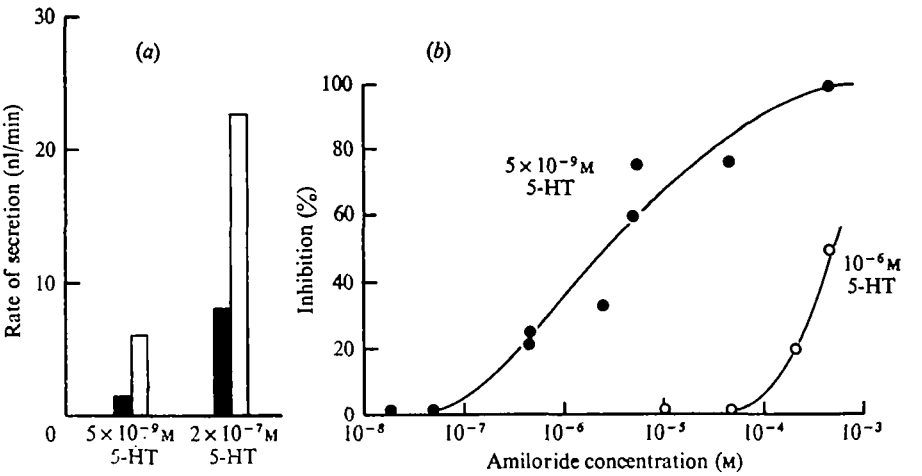


Fig. 8. (*a*) The effect of raising the extracellular concentration of calcium from 0·1 mM (solid bars) to 5 mM (open bars) on the inhibitory effect of $2 \times 10^{-4}$ M amiloride at two different 5-HT concentrations. (*b*) the ability of 5-HT to overcome amiloride inhibition. The inhibitory effect at $10^{-6}$ M 5-HT (○) was much less than that observed at $5 \times 10^{-9}$ M 5-HT (●). In these experiments the calcium concentration was 0·1 mM.

in detail elsewhere (Berridge, Lindley & Prince, 1975*b*). In a potassium-free saline where the glands are secreting sodium (Fig. 1*b*), amiloride caused a complete inhibition of secretion (Fig. 7*b*).

The inhibitory effect of amiloride can be overcome by raising the concentrations of either calcium or 5-HT (Fig. 8). Increasing the calcium concentration from 0·1 mM to 5 mM causes a threefold increase in the rate of secretion at two different 5-HT

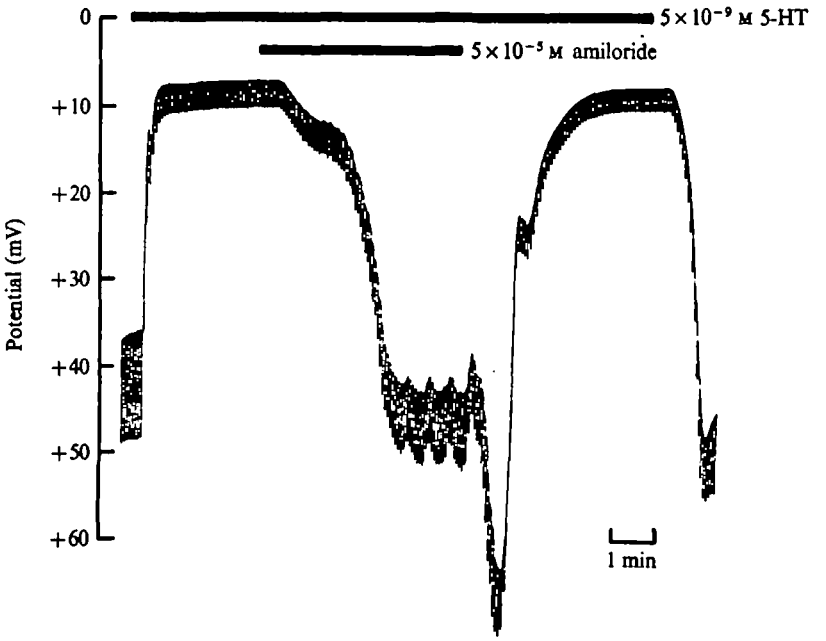


Fig. 9. The effect of $5 \times 10^{-5}$ M amiloride on the transepithelial potential and resistance of salivary glands during stimulation with $5 \times 10^{-9}$ M 5-HT. The potential deflexions, which are a measure of the resistance, were caused by regular current pulses of 0·14 μA.

concentrations (Fig. 8*a*). The inhibitory effect of $2 \times 10^{-4}$ M amiloride was much less at the higher 5-HT concentration. The protective effect of raising the concentration of 5-HT is illustrated in Figure 8*b*. At $5 \times 10^{-9}$ M 5-HT half maximal inhibition of secretion was produced by $2 \times 10^{-6}$ M amiloride but much more amiloride was required ($5 \times 10^{-4}$ M) to produce the same effect when the glands were stimulated with $10^{-6}$ M 5-HT.

The effect of amiloride on potential and resistance is shown in Fig. 9. When 5-HT was applied the transepithelial potential (lumen relative to bathing medium) went negative and there was a marked decrease in resistance as shown by the decrease in the potential deflexions produced by applying regular current pulses. Previous studies have shown that both these changes are mediated by calcium (Prince & Berridge, 1973; Berridge *et al.* 1975*a*). Amiloride caused both the potential and the resistance to return towards the resting value. On removal of amiloride, the potential went rapidly positive before returning to the previous stimulated level.

## DISCUSSION

*Calliphora* salivary glands, like other epithelia, may be considered to consist of two membranes arranged in series. To understand how 5-HT stimulates fluid secretion it is necessary to understand the ionic events taking place across both the basal and apical surfaces. The following evidence indicates that the 'prime mover' for the increase in fluid secretion by salivary glands is an increase in potassium transport. Potassium is transported uphill with the gland showing a marked preference for potassium over

sodium (Fig. 1*b*). Potential responses to 5-HT in the absence of a permeable anion indicate that the apical membrane has an active cation pump which may be electrogenic (Berridge & Prince, 1972*a*). These potential responses are inhibited by dinitrophenol, cyanide and ethacrynic acid, compounds which inhibit active transport in other tissues (Prince, 1971). Ramsay (1953) first recognized the central role of potassium transport during fluid secretion by insect Malpighian tubules. Subsequent studies have confirmed the importance of potassium not only in Malpighian tubules (Berridge, 1968; Maddrell, 1969) but also in the midgut of insects (Harvey & Nedergaard, 1964) as well as in these salivary glands. The potassium pump of insects appears to be relatively unspecific and will readily transport other cations under appropriate conditions (Berridge, 1968; Zerahn, 1971; Maddrell, unpublished observations). This lack of specificity suggests that the cation which is transported is determined by the major cation present within the intracellular compartment which, in turn, is determined by the permeability of the basal plasma membrane rather than by the affinity of the pump for a particular ion. So far it has not been possible to determine the intracellular potassium concentration directly, but, since the Nernst slope (Fig. 3*a*) during 5-HT stimulation is 53 mV for a tenfold change in external potassium and the intercept on the $x$ axis is 190 mM, the glands must have a high internal potassium concentration. However, when potassium was removed from the bathing medium the glands secreted sodium (Fig. 1*b*). Also rubidium was able to support secretion in the absence of potassium (Table 1). Presumably under such conditions the intracellular potassium concentration drops and the concentration of other cations rises so that they now have access to the apical pump sites. A changeover of the intracellular pool from potassium to sodium may account for the response of the basal membrane when the glands were transferred to a potassium-free medium (Fig. 5). Initially there was the expected large hyperpolarization of the basal membrane but this was not maintained and the potential drifted back to the initial value, presumably as the intracellular potassium was partially replaced with sodium. Although subsequent potential responses to 5-HT were qualitatively similar to those seen in normal saline, the hyperpolarization across the basal membrane was considerably exaggerated (Fig. 5). Perhaps this arises because the permeability of the basal membrane to sodium is low. If cation entry into the cell is controlled by the chloride gradient, the larger hyperpolarization seen in sodium media may arise because the internal chloride level must be driven much lower than in normal saline to create a sufficient gradient to drag sodium into the cell fast enough to satisfy the cation pump on the apical membrane. Another indication that the sodium permeability is low across the basal membrane is apparent in Fig. 2 where the rate of secretion was linearly related over a wide range of external sodium concentration (0–150 mM) whereas maximum secretion is obtained with low concentrations of potassium (10 mM).

The potential responses to 5-HT which we observe across the basal membrane are consistent with this membrane being permeable to potassium at rest and becoming more permeable to both potassium and chloride during stimulation. The Nernst plots for potassium under various conditions attempted to verify these permeability changes (Fig. 3). The Nernst plot in the presence of 5-HT (53 mV) is closer to the theoretical value of 58 mV (for a truly potassium permselective membrane) than that obtained in the absence of 5-HT (44 mV), suggesting that 5-HT increases potassium permeability.

However, this explanation may not be completely valid because the marked hyperpolarization observed at low potassium concentrations was abolished when chloride in the bathing medium was replaced with isethionate (Fig. 4*b*). In normal saline we suppose that hyperpolarization of the basal membrane is the result of a decrease in the intracellular concentration of chloride (Berridge *et al.* 1975*a*). This is caused by the movement of chloride across the apical membrane following the potassium which is pumped into the lumen. The hyperpolarization may provide the necessary gradient to increase the influx of potassium and chloride into the cell. Thus, in low potassium solutions, the decrease in the intracellular chloride concentration, and hence the resultant hyperpolarization, must be larger to overcome the effect of the increased potassium gradient. The Nernst plots may be explained by the cooperative effect of a change in resistance to chloride across the basal membrane, as observed previously (Berridge *et al.* 1975*a*), in conjunction with the effect of the apical pump on intracellular chloride concentration. If this is correct then either removal of chloride or inhibition of the apical pump should inhibit the hyperpolarization of the basal membrane and this is exactly what is seen experimentally (Fig. 4*b* and Prince, 1971). These results emphasize that potential responses in these salivary glands are determined by a delicate balance between the activity of the apical pump and the supply of ions which is governed by the permeability of the basal membrane.

Amiloride may inhibit fluid secretion by interfering with the movement of cations across the basal plasma membrane. In toad bladder and frog skin amiloride is thought to inhibit the passive entry of sodium across the mucosal surface (Bentley, 1968; Cuthbert & Wong, 1972). In the case of these salivary glands, however, there are indications that it may inhibit the influx of calcium although we have not yet studied this directly. Its effect on transepithelial potential and resistance are very similar to those seen in glands stimulated with 5-HT in calcium-free media (Prince & Berridge, 1973; Berridge *et al.* 1975*a*). The inhibitory effect of amiloride was partially overcome both by raising the external calcium concentration (Fig. 8*a*) as well as by raising the concentration of 5-HT (Fig. 8*b*). The last effect may depend on the ability of 5-HT to stimulate the synthesis of cyclic AMP which, in turn, may release calcium from intracellular reservoirs (Berridge & Prince, 1972*b*; Prince & Berridge, 1973). By blocking the influx of calcium, amiloride will reduce the permeability of the basal and apical membranes to chloride thus inhibiting the normal secretory and potential responses.

In summary, the mechanism of secretion by *Calliphora* salivary glands can be explained on the basis of an active electrogenic cation pump situated on the apical membrane which entrains a parallel flow of chloride to maintain electroneutrality. The potential across this membrane is critically dependent on the supply of chloride entering across the basal membrane which, in turn, is regulated by the intracellular level of calcium. When secretion is stimulated, the permeability of the basal membrane appears to increase to both potassium and chloride to enable these ions to enter the cell fast enough to compensate for their rapid removal from the cell into the lumen. Although all our observations are consistent with chloride movement being passive, we cannot completely exclude the possibility of a pump on this basal membrane which transports chloride into the cell. The results in this paper demonstrate that the changes in potential in a transporting epithelium must be analysed not only in terms of changes in ion pumps and membrane permeabilities (to both cations and anions)

but also in terms of changes in the concentration of the intracellular pool of ions such as chloride.

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