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Relationship between Hormonal Activation of Phosphatidylinositol Hydrolysis, Fluid Secretion and Calcium Flux in the Blowfly Salivary Gland

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The addition of 5-hydroxytryptamine to the isolated blowfly salivary gland stimulates fluid secretion, transepithelial calcium transport and the breakdown of ^{32}P- or ^{3}H-labelled phosphatidylinositol. The breakdown of [^{32}P]phosphatidylcholine and [^{32}P]-phosphatidylethanolamine was not stimulated by 5-hydroxytryptamine. In salivary glands incubated with *myo*-[2-^{3}H]inositol for 1–3 h, more than 95% of the label retained by the tissue was in the form of phosphatidylinositol. The addition of 5-hydroxytryptamine resulted in an increase in the accumulation of label in intracellular inositol 1:2-cyclic phosphate, inositol 1-phosphate and free inositol along with an increase in the release of [^{3}H]inositol to the medium and saliva. The release of [^{3}H]inositol to the medium served as a sensitive indicator of phosphatidylinositol breakdown. The release of [^{3}H]inositol was not increased by cyclic AMP or the bivalent-cation ionophore A23187 under conditions in which salivary secretion was accelerated. The stimulation of fluid secretion by low concentrations of 5-hydroxytryptamine was potentiated by 3-isobutyl-1-methylxanthine, which had no effect on inositol release. The stimulation of fluid secretion by 5-hydroxytryptamine was greatly reduced in calcium-free buffer, but the breakdown of phosphatidylinositol continued at the same rate in the absence of calcium. These results support the hypothesis that breakdown of phosphatidylinositol by 5-hydroxytryptamine is involved in the gating of calcium.

A variety of hormonal stimuli, particularly muscarinic cholinergic and α-adrenergic stimulation, result in an increased turnover of phosphatidylinositol in many mammalian tissues (Hawthorne, 1973; Michell *et al.*, 1976, 1977*a*,*b*). It has been proposed that the hydrolysis of phosphatidylinositol induced by a hormone–receptor complex might be directly involved in controlling calcium entry into cells (Michell, 1975; Michell *et al.*, 1976, 1977*a*,*b*). This hypothesis is based mainly on the observation that in tissues in which hormones are thought to increase the entry of extracellular calcium there is an increased turnover of phosphatidylinositol. To establish the relationship between phosphatidylinositol hydrolysis and calcium flux more fully, we have studied these two parameters in the isolated insect salivary gland.

The salivary glands of the adult blowfly are long thin tubes made up of a single layer of homogeneous cells, which secrete iso-osmotic KCl when stimulated by 5-hydroxytryptamine (Berridge & Prince, 1973; Berridge, 1975). The bivalent-cation ionophore A23187 can stimulate fluid secretion and there is also a dependence on extracellular calcium for continued secretion in response to 5-hydroxytryptamine (Berridge, 1975). Flux studies with $^{45}Ca^{2+}$ have shown that an important action of 5-hydroxytryptamine is to stimulate the entry of calcium (Prince *et al.*, 1972; Berridge & Lipke, 1979). Much of the calcium which enters the cells is extruded into the saliva, thus establishing a transepithelial flux of calcium, which provides a simple method for measuring calcium permeability (Berridge & Lipke, 1979). Since the entry of calcium into the cell is the rate-limiting step, it is possible to use the rate of calcium transport across the whole gland as a method for continuously monitoring calcium permeability. The present studies were designed to determine whether the increased entry of calcium seen after the addition of 5-hydroxytryptamine was accompanied by an increase in phosphatidylinositol hydrolysis. A preliminary report has been presented (Fain & Berridge, 1978).

Methods

Incubation conditions

Salivary glands were isolated from adult female blowflies (*Calliphora erythrocephala*). The age of the flies used ranged from 5 to 10 days after emergence; they were maintained at 15–20°C on a diet of sucrose, pig's heart and water. The buffer used for dissection and incubation of the salivary glands had the following composition (mM): Na^+, 155; K^+, 10; Ca^{2+}, 2; Mg^{2+}, 2; Tris, 10; Cl^-, 156; malate, 2.7; glutamate,

2.7; glucose, 10. The pH was adjusted to 7.3 with Tris/HCl. All experiments were conducted at room temperature (15–20°C). If fluid secretion and calcium transport were to be measured, the glands were set up in 50 μl of medium under liquid paraffin and their cut ends were pulled a short distance out into the liquid paraffin by means of single silk threads. The glands were nicked immediately behind the ligature and the saliva accumulated as a separate drop around this cut end. The volume of fluid secreted during set intervals was determined from microsopic measurement of the diameter of the drop.

To measure calcium transport, $^{45}CaCl_2$ (specific radioactivity 12.7 Ci/mg; The Radiochemical Centre, Amersham, Bucks., U.K.) was added to the medium bathing the glands and the rate at which radioactivity appeared in the saliva was measured. The drops of saliva were removed from under the liquid paraffin and transferred to counting vials by Pasteur pipettes.

Inositol release from the cells was measured by first adding *myo*-[2-^{3}H]inositol (see the next section for details) to the medium bathing isolated glands that had been ligated as described above. After this labelling period, the glands were thoroughly washed with inositol-free medium before the start of the efflux experiment. The release of [^{3}H]inositol to the saliva was determined by removing the drops from under the liquid paraffin at set intervals with Pasteur pipettes and adding the drops to counting vials. At similar intervals, the entire medium surrounding the glands was removed and counted for radioactivity while fresh medium was added to the glands to measure efflux over the next interval.

Labelling and extraction procedure for phospholipids

The glands were labelled by incubation with a tracer amount of [^{32}P]P_i (sp. radioactivity 100–150 Ci/mg of P in dilute HCl, pH 2–3) and about 3×10^7 c.p.m./0.5 ml of medium was used for each experiment. In studies with *myo*-[2-^{3}H]inositol (sp. radioactivity 5 Ci/mmol; The Radiochemical Centre), two to four glands were incubated in 50 μl of medium containing 1.05×10^6 c.p.m., which gave an inositol concentration of 5 μM, or 4.2×10^6 c.p.m. for an inositol concentration of 20 μM. Under these conditions the incorporation of 4200 c.p.m. represents the formation of 1 pmol of phosphatidylinositol.

The incubation with label was terminated by the removal of the medium and the glands were rinsed five times with fresh medium over a 15–30 min period. This washing procedure removed nearly all the labelled inositol that was not present in phosphatidylinositol. If the glands were homogenized with 0.2 ml of 7% (w/v) $HClO_4$, the amount of acid-soluble label was 6.5 $\pm$ 0.4% (S.E.M. for 12 experiments) of the label present in phospholipids. Phospholipids were extracted by homogenization of the glands in 0.5 ml of chloroform/methanol (2:1, v/v). The procedure was repeated and the phases were then separated by the addition of 0.5 ml of water.

Chromatographic separation of inositol and water-soluble metabolites

If the glands were homogenized directly in chloroform/methanol, the inositol and water-soluble metabolites present in salivary glands were recovered in the upper phase after addition of water. A sample of the latter was reduced under vacuum and then applied to Whatman SG 81 paper impregnated with 22% silica gel (silicon dioxide) to separate these water-soluble compounds. The chromatograms were developed (ascending) with chloroform/methanol/25% (v/v) NH_3 soln. (6:10:5, by vol.) for 3–4 h (Koch-Kallnbach & Diringer, 1977). The chromatograms were stained with $KMnO_4$ (1.0%) containing 2% (w/v) Na_2CO_3 for location of inositol and other compounds.

Glycerophosphoinositol was obtained by mild alkaline hydrolysis of the labelled phosphatidylinositol extracted from the salivary glands by the procedure of Dawson (1960). Inositol 1:2-cyclic phosphate and a mixture of inositol 1-phosphate and inositol 2-phosphate were prepared as described by Pizer & Ballou (1959). The material moving in the same position as inositol 1:2-cyclic phosphate was converted into inositol monophosphates by hydrolysis with 0.1 M-HCl for 5 min at 80°C. The R_F values were: inositol 1-phosphate, 0.1; inositol, 0.4; glycerophosphoinositol, 0.5; inositol 1:2-cyclic phosphate, 0.9. The separation of these compounds was also examined by using glass-fibre sheets impregnated with silica gel or poly(silicic acid) gel (Hokin-Neaverson & Sadeghian, 1976). The percentage of water-soluble radioactivity present as free inositol measured by using this silica-gel-loaded paper was checked by ion-exchange chromatography on Dowex-1 (formate form) columns set up in Pasteur pipettes (4mm $\times$ 16mm).

Chromatographic separation of phospholipids

Analysis of samples of the chloroform layer indicated only 66% recovery of labelled phosphatidylinositol in this phase. The remainder of the labelled lipid was associated with protein present at the interface between the two layers formed after addition of water to separate the phases. Palmer (1971) first reported that phosphatidylinositol binds to protein present at the interface. The use of 2 M-KCl or -NaCl to separate the phases lessens this problem (Palmer, 1971). We circumvented this difficulty by using the entire lower phase (chloroform layer) plus the interface containing protein-bound phosphatidyl-

inositol, which is evaporated to dryness after washing three to five times with equilibrated upper phase.

The lipids were redissolved in chloroform/methanol (3:1, v/v) and separated by the procedure of Rouser *et al.* (1970) as modified by R. G. Bridges (unpublished work), with two-dimensional t.l.c. on 20cm × 20cm glass plates coated with silica gel H (0.25mm thick) containing magnesium acetate (1g/40g of silica gel). In this procedure, phosphatidylserine is easily separated from phosphatidylinositol, as is phosphatidylglycerol from phosphatidylethanolamine. The plates were run in the first dimension for 1.5h in chloroform/methanol/water/4.3M-NH_3 (26:14:1:1, by vol.). The plates were removed, dried briefly in a hood with the aid of an electric hair-dryer and then the remaining solvent was removed by vacuum desiccation for at least 2h. The plates were run in the second dimension in chloroform/methanol/acetic acid/water (75:25:7:3, by vol.) for 1–1.5h. The plates were removed and the location of phospholipids was determined by using iodine-vapour staining. If small amounts of phospholipids (less than 30 glands) were analysed, it was necessary to add carrier phospholipids to permit detection of the compounds. Radioactive phospholipids were located by radioautography for 48–56h by using X-ray film or by scanning with a Berthold Thin-Layer Scanner II for ^{32}P-labelled phospholipids.

There was no detectable incorporation of [^{32}P]P_i or evidence from iodine staining for the presence of phospholipids other than those shown in Table 1. We could not detect any CDP-diacylglycerol or lysophospholipids in our salivary-gland extracts. The location of all identified phospholipids was based on the separation of phospholipid standards. The phospholipid denoted as 'X' is probably ceramide phosphoethanolamine, which has a similar mobility and is present in blowflies (Dawson & Kemp, 1968). Crone & Bridges (1963) originally reported the presence in houseflies of a phospholipid with properties similar to those of ceramide phosphoethanolamine.

In experiments in which the uptake of label into di- and tri-phosphatidylinositol was determined, the glands were extracted with chloroform/methanol (2:1, v/v) containing 0.25% HCl. The phosphorus content of phospholids was determined by a micro modification of the procedure of Bartlett (1959) after acid hydrolysis of silica-gel scrapings containing each individual phospholipid. Samples of the hydrolysate were also counted to determine the amount of radioactivity present in each phospholipid as described below.

Radioactivity counting procedures

The ^3H present in scrapings from thin-layer plates or sections from paper chromatograms was counted by addition of 0.2ml of 50% (v/v) methanol followed by 4ml of Biofluor (New England Nuclear Corp., Boston, MA, U.S.A.) to the samples in a plastic mini-scintillation vial. Samples of the chloroform/methanol extracts were also counted by the same procedure after removal of the chloroform, which decreases the radioactivity counting efficiency if not removed. *myo*-[2-^3H]Inositol and $^{45}Ca^{2+}$ present in medium and saliva were determined by adding the samples to mini-vials containing 4ml of Biofluor. The counting efficiency for samples of medium or saliva (up to 50μl) was the same as that for phospholipids in Biofluor. There was a 20% decrease when ^3H from the silica-gel scrapings was counted, and a 50% decrease for silica-gel paper. Radioactivity was measured by using dual-channel liquid-scintillation counters with a counting efficiency for ^3H in Biofluor of 33%, and standard procedures were used for determination of [^{32}P]P_i and ^3H in the same samples.

Reproducibility of results

Although sometimes representative results from a single experiment are presented here, where effects were small or there was considerable variability from one experiment to the next we have presented data from up to 14 experiments. Except as noted the results are from a representative experiment. Reproducibility of results from independent biological experiments was excellent for measurements of salivary secretion, Ca^{2+} flux and inositol release. Generally, key experiments were repeated at least twice for the result shown in the Figures but often under slightly different conditions.

Results

Phospholipid composition of salivary glands and the uptake of [^{32}P]P_i *into phospholipids*

Phosphatidylcholine and phosphatidylethanolamine were the major phospholipids found in the blowfly salivary gland and together accounted for 51% of the phospholipid content (Table 1). There were lesser amounts of phosphatidylserine, phosphatidylglycerol, cardiolipin, phosphatidylinositol and the phospholipid 'X'. The phosphatidylinositol content was approx. 140pmol/gland based on phosphorus content.

The incorporation of [^{32}P]P_i into phospholipids in the absence of added inositol is shown in Table 1. There was little incorporation of label into cardiolipin or phosphatidylserine. Formation of labelled phosphatidylethanolamine and phosphatidylcholine accounted for about half the total incorporation of label. The amount of label present in phosphatidylinositol was 8% of the total incorporation into phospholipids, and phosphatidylglycerol formation

accounted for 29% of the total incorporation (Table 1). In a separate experiment, the incorporation of [^{32}P]P_i was also measured in the presence of unlabelled 2mM-inositol. The total incorporation of label was unaffected, but phosphatidylinositol formation now accounted for 30% of the label incorporated into

Table 1. *Phospholipid composition of fly salivary glands*

For this, 36 salivary glands were incubated for 2h in 0.5ml of medium containing a tracer amount of [^{32}P]P_i (3×10^7c.p.m.). At the end of the incubation the glands were homogenzied in cholorform/methanol (2:1, v/v) and the phospholipids present in the chloroform phase were separated by two-dimensional t.l.c. The values are the means for six experiments.

	Phospholipid content		Phospholipid radioactivity		Sp. radioactivity of phospholipids
Phospholipid	(pmol of P/gland)	(% of total content)	(c.p.m./gland)	(% of total label)	(c.p.m./100pmol of P)
Phosphatidate	113	7	85	4	75
Phosphatidylglycerol	114	7	600	29	526
Phosphatidylinositol	139	8	176	8	127
Phosphatidylcholine	360	21	580	28	161
'X'	143	8	280	13	195
Phosphatidylethanolamine	495	30	350	16	71
Phosphatidylserine	117	7	17	1	14
Cardiolipin	200	12	22	1	11

Table 2. *Effect of 5-hydroxytryptamine on breakdown of labelled phospholipids*

Four salivary glands were first incubated for 30min with [^{32}P]P_i and *myo*-[2-^{3}H]inositol (20μM). The glands were then incubated for 3.5h in buffer with 7mM-phosphate and 1mM-inositol before the experiment in which the glands were incubated for 2h with or without 1μM-5-hydroxytryptamine. The values are the means ± S.E.M. for three paired experiments.

	Control	+1μM-5-Hydroxytryptamine	
	(c.p.m.)	(c.p.m.)	(% change caused by 5-hydroxytryptamine)
[^{3}H]Inositol label in			
Diphosphoinositide and triphosphoinositide	2800	615	−78 ± 6
Phosphatidylinositol	24660	565	−77 ± 5
[^{32}P]P_i label in			
Di- and tri-phosphatidylinositol plus phosphatidylserine	2000	400	−80 ± 10
Phosphatidylinositol	18300	5850	−68 ± 4
Phosphatidate	1010	605	−40 ± 12
Phosphatidylglycerol	5870	4345	−26 ± 5
Phosphatidylcholine	6600	6140	−7 ± 30
Phosphatidylethanolamine	4000	4600	+15 ± 50

Table 3. *Intracellular accumulation of inositol label in the presence of 5-hydroxytryptamine*

Four salivary glands were incubated for 2h with 20μM-*myo*-[2-^{3}H]inositol and then washed five times over a period of 30min. The amount of label present in phosphatidylinositol was approx. 42000 c.p.m. The glands were then incubated for 1min with or without 10μM-5-hydroxytryptamine. The glands were extracted with chloroform/methanol (2:1, v/v) and the label present in the upper phase was chromatographed to separate the water-soluble compounds. The release of [^{3}H]inositol to the medium was 158c.p.m in the absence and 360c.p.m. during the first minute after the addition of 10μM-5-hydroxytryptamine. The values are the means for 14 paired experiments and the percentage increases are the means ± S.E.M. for the paired differences. Statistical significance was determined by using the *t* test based on Fisher's *t* distribution.

		+10μM-5-Hydroxytryptamine	
Label present in glands as	Control (c.p.m.)	(c.p.m.)	(% change caused by 5-hydroxytryptamine)
Inositol	200	276	+38 ± 14*
Inositol 1-phosphate	76	161	+112 ± 33**
Inositol 1:2-cyclic phosphate	574	826	+44 ± 13**
Glycerophosphoinositol	280	308	+10 ± 24

* $P<0.05$.
** $P<0.01$.

phospholipids and phosphatidylglycerol formation was decreased to 9% of the total.

Effect of 5-hydroxytryptamine on the breakdown of phospholipids prelabelled with myo-[2-^3H]inositol or [^{32}P]P_i

We examined the effect of 5-hydroxytryptamine on the breakdown of phospholipids prelabelled with either *myo*-[2-^{3}H]inositol or [^{32}P]P_i. After labelling for 30min, the glands were incubated for 3.5h to ensure equilibration of newly synthesized phosphatidylinositol; the medium contained 7mM-phosphate and 1mM-inositol to decrease further synthesis of labelled phospholipids. If the glands were then incubated with 1μM-5-hydroxytryptamine for 2h, there was a large loss of label from phosphatidylinositol, but no loss of label from phosphatidylcholine or phosphatidylethanolamine (Table 2). There was some loss of label from phosphatidate and phosphatidylglycerol. The effects of 5-hydroxytryptamine on the disappearance of [^{32}P]P_i or [^{3}H]inositol label were similar, which suggests that phosphatidylinositol was being degraded to diacylglycerol and inositol phosphates, which were subsequently cleaved to inositol and P_i. In the experiments shown in Table 2, the glands were homogenized with acidified chloroform/methanol to extract di- and tri-phosphoinositides. However, they accounted for less than 10% of the total label taken up by the phosphoinositides, and their breakdown was affected to the same extent as that of monophosphatidylinositol. In subsequent experiments we extracted the glands with chloroform/methanol (2:1, v/v) and only examined the turnover of phosphatidylinositol.

There was close correspondence between the decline in [^{3}H]inositol label in phospholipids (18000c.p.m.) and the recovery of [^{3}H]inositol in the medium (22000c.p.m.) during stimulation by 5-hydroxytryptamine in the experiment summarized in Table 2. With [^{32}P]P_i, however, the decrease in label in all phospholipids was 16000c.p.m., whereas the increase in appearance of label in the medium was 66000c.p.m., suggesting that [^{32}P]P_i was being released from sources other than the phospholipids. To explore the possibility that the rate at which labelled inositol is released to the medium reflects the rate at which phosphatidylinositol is being hydrolysed, we studied the effect of 5-hydroxytryptamine on some of the expected intermediates of phosphatidylinositol metabolism.

Effect of 5-hydroxytryptamine on the release and cellular content of inositol, inositol phosphates and glycerophosphoinositol

Measuring the decrease of labelled phosphatidylinositol directly is not a very sensitive assay for hormone-induced breakdown because of the difficulty in measuring the loss of small amounts of label. A much more sensitive assay for breakdown is to monitor the appearance of label in breakdown products of phosphatidylinositol hydrolysis.

The release of radioactivity to the saliva or medium in the absence or presence of 5-hydroxytryptamine was solely as [^{3}H]inositol (results not shown). However, in the gland only 17% of the water-soluble radioactivity was free inositol. From the data in

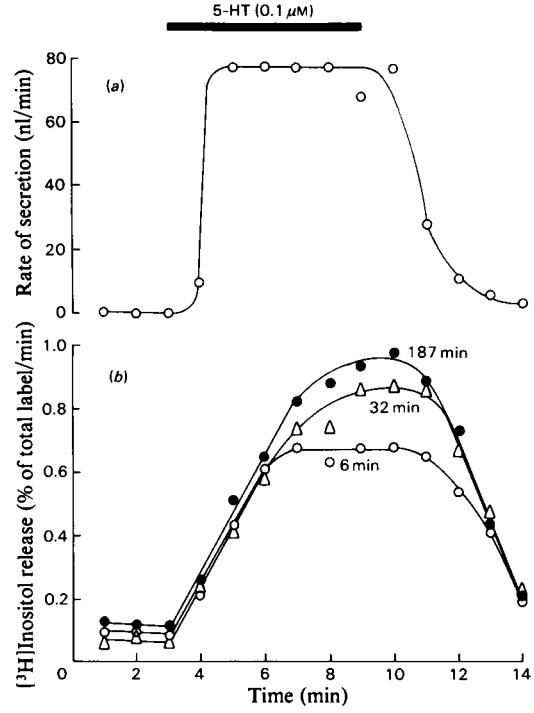

Fig. 1. *Influence of time after labelling on the percentage breakdown of phosphatidylinositol caused by 5-hydroxytryptamine*

Four salivary glands were incubated with *myo*-[2-^{3}H]inositol (20μM) for 2h. After the label was washed away, the glands were incubated in control medium. At 6min (○), 32min (△) and 187min (●) after removal of the labelling solution the release of [^{3}H]-inositol to the medium plus saliva was measured at 1min intervals for 14min (*b*). The amount of [^{3}H]-inositol released during each minute is expressed as percentage of the amount of labelled phosphatidylinositol present in the gland at that time. The glands were exposed to 0.1μM-5-hydroxytryptamine (5-HT) for a 6min test period during the period indicated by the bar. The increase in the rate of fluid secretion (*a*) during each 5-hydroxytryptamine treatment was almost identical and for clarity only the response during the first 5-hydroxytryptamine stimulation is illustrated. The data are from a single experiment.

Table 3, the distribution of label in control glands was as follows: inositol 1:2-cyclic phosphate (51%), inositol 1-phosphate (7%), glycerophosphoinositol (25%) and free inositol (17%). The effect of 5-hydroxytryptamine on the amount of intracellular label was variable, but there were significant increases in labelled inositol 1:2-cyclic phosphate, inositol 1-phosphate and free inositol 1 min after the addition of 10 μM-5-hydroxytryptamine (Table 3). These results suggest that the breakdown of phosphatidylinositol occurs by the action of a phospholipase, whose products are diacylglycerol and inositol 1:2-cyclic phosphate, as originally shown by Dawson *et al.* (1971).

The free inositol pool in the salivary gland is small. Over a 1 min period, an unstimulated gland releases an amount of [^{3}H]inositol equivalent to 80% of its intracellular pool of free [^{3}H]inositol. The latter increased rather than decreased during the action of 5-hydroxytryptamine (Table 3). Therefore the increased release of inositol label from the glands during the action of 5-hydroxytryptamine must result from an increase in breakdown of labelled phosphatidylinositol.

Variation in the nature of inositol release with time after labelling

Newly synthesized [^{3}H]phosphatidylinositol appeared to equilibrate rapidly with unlabelled phosphatidylinositol susceptible to the action of 5-hydroxytryptamine. If a salivary gland was labelled by incubation for 1 h with *myo*-[2-^{3}H]-inositol (20 μM) between 1.5 and 4 pmol of phosphatidylinositol/gland was formed. The ability of 0.1 μM-5-hydroxytryptamine to stimulate the efflux of label from such glands was then studied at various times after removing the label (Fig. 1). If the glands

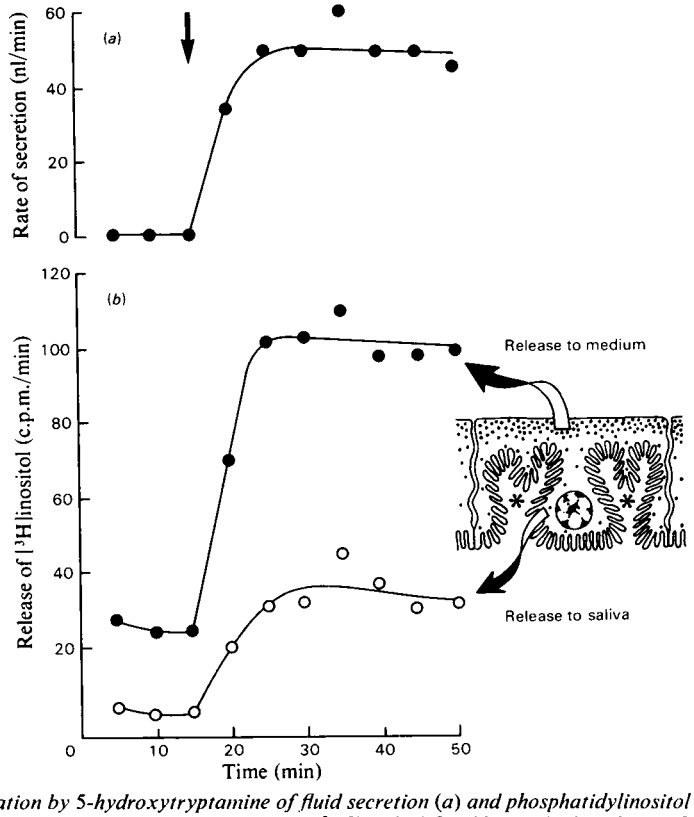

Fig. 2. *Stimulation by 5-hydroxytryptamine of fluid secretion (a) and phosphatidylinositol breakdown (b)*
Four salivary glands were incubated with 5 μM-*myo*-[2-^{3}H]inositol for 2 h, washed and transferred to fresh medium without label for 15 min before starting the efflux measurements. 5-Hydroxytryptamine (0.1 μM) was added after 15 min (arrow) and the release of inositol to medium (●) and saliva (○) are shown separately (*b*). The illustration at the right shows the major structural features of a secretory unit in the fly salivary gland. Note the large increase in surface area of the apical membrane, which is arranged in the form of canaliculi (*) opening in the direction where saliva is secreted into the lumen.

were stimulated 6min after coming out of the label, there was a significant increase in the efflux of [^{3}H]inositol during the action of 5-hydroxytryptamine. Allowing the glands to incubate in control medium for 32min and 187min produced slightly larger increases in the amount of [^{3}H]inositol released during the action of 5-hydroxytryptamine but there was little change either in the onset of, or in the recovery from, the response (Fig. 1). The onset of [^{3}H]inositol efflux coincided with the onset of fluid secretion, but the rate of [^{3}H]inositol efflux continued to increase after fluid secretion had reached a maximum. These results indicate that newly synthesized phosphatidylinositol appears rapidly in the pool, where it becomes susceptible to the action of 5-hydroxytryptamine.

Comparison of the effects of 5-hydroxytryptamine and cyclic AMP on phosphatidylinositol hydrolysis and calcium transport

In the first series of experiments, we studied the effect of stimulating glands continuously with 0.1 μM-5-hydroxytryptamine (Fig. 2). The rate of release of [^{3}H]inositol to both the medium and saliva rapidly increased and remained at a steady value as long as 5-hydroxytryptamine was present (35min). As noted in Fig. 1, the onset for [^{3}H]inositol release was similar to that for the increase in fluid secretion. Most of the release is to the medium rather than to the saliva, despite the much larger surface area of the apical (luminal) membrane (Oschman & Berridge, 1970).

If calcium transport results from an increase in phosphatidylinositol hydrolysis, these two parameters should be related to each other under a variety of experimental conditions. We studied the effect of varying the concentration of 5-hydroxytryptamine on the rate of release of inositol (Fig. 3). A small increase in inositol release was seen with 0.001 μM-5-hydroxytryptamine (Fig. 3). The higher concentrations of 5-hydroxytryptamine resulted in larger release rates, which remained relatively constant for concentrations of 5-hydroxytryptamine up to 0.1 μM (as noted in Fig. 2). There was a decline in release with time with 1 μM-5-hydroxytryptamine, which was probably due to a depletion of labelled phosphatidylinositol. The data in Table 2 indicate that after a 2h incubation with 1 μM-5-hydroxytryptamine 80% of the labelled phosphatidylinositol had been hydrolysed.

A similar range of 5-hydroxytryptamine concentrations was used for the calcium-transport study (Fig. 4). In this experiment, the different concentrations of 5-hydroxytryptamine were superimposed on a background of 10mM-cyclic AMP (plus 1mM-theophylline), which by itself had very little effect on calcium transport (Fig. 4), even though it was able to stimulate fluid secretion almost maximally (20nl/min). The fact that fluid secretion was maximally activated by the presence of cyclic AMP ensured that any effects of hormones on $^{45}Ca^{2+}$ transport were not secondary to fluid secretion. The addition of 0.001 μM-5-hydroxytryptamine produced a small further increase in salivary flow to 27nl/min and also increased the flux of $^{45}Ca^{2+}$ across the gland. Further increases in 5-hydroxytryptamine concentration had no greater effect on salivary flow, but they substantially increased the transport of calcium in a

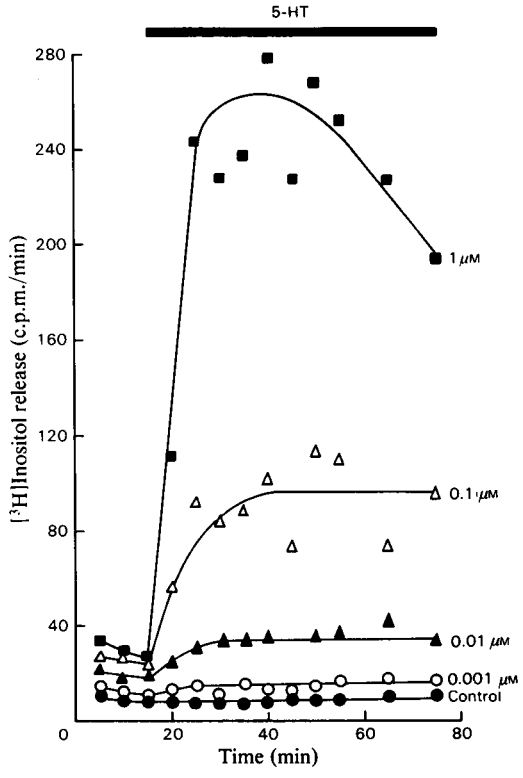

Fig. 3. *Stimulation of [^{3}H]inositol release by varying the concentration of 5-hydroxytryptamine*

Groups of four salivary glands were incubated with 5 μM-*myo*-[2-^{3}H]inositol for 2h, washed and transferred to fresh medium without label for 15min. The data are shown as release of label to the medium in the absence (●) or presence of 5-hydroxytryptamine at the following concentrations: 0.001 μM (○), 0.01 μM (▲), 0.1 μM (△) and 1 μM (■). The 5-hydroxytryptamine (5-HT) was added after 15min (solid bar) and was present for the remainder of the incubation. At all time periods the release of label to the saliva was about one-third of that released to the medium. Samples were taken every 5min and the rate of release of [^{3}H]inositol is expressed as c.p.m./min per gland for a single experiment.

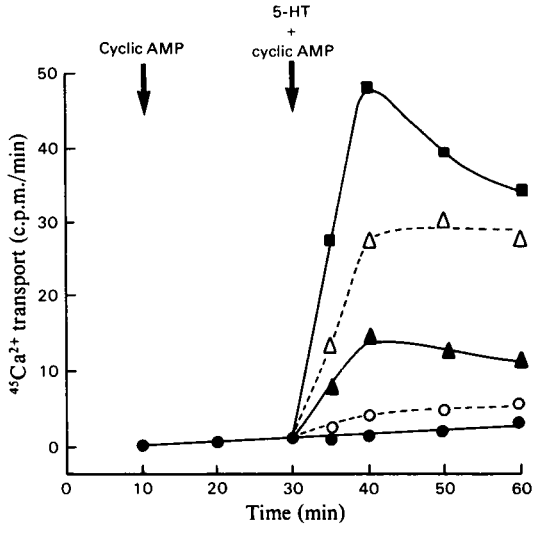

Fig. 4. *Stimulation of transepithelial calcium transport by 5-hydroxytryptamine*

Groups of two salivary glands were incubated for 60 min in buffer containing 7000 c.p.m. of $^{45}Ca^{2+}/\mu l$. Cyclic AMP at a concentration of 10 mM (plus 1 mM-theophylline) was present from the start of the incubation. The data represent $^{45}Ca^{2+}$ transfer from the medium to saliva in c.p.m./min for samples taken every 5 min from each gland incubated with cyclic AMP plus theophylline aline (●), or with 5-hydroxytryptamine (5-HT): 0.001 μM (○), 0.01 μM (▲), 0.1 μM (△) or 1 μM (■).

dose-dependent manner (Fig. 4). At the highest concentration of 5-hydroxytryptamine (1 μM), the rate of calcium transport reached a peak 10 min after 5-hydroxytryptamine stimulation and then began to fall. This inactivation of calcium entry is described in more detail in the accompanying paper (Berridge & Fain, 1979).

The effects of varying the concentration of 5-hydroxytryptamine on both inositol release and calcium transport are illustrated in Fig. 5. Half-maximal stimulation of fluid secretion, calcium transport and inositol release required 0.003 μM-, 0.02 μM- and 0.30 μM-5-hydroxytryptamine respectively. A maximal rate of fluid secretion was observed over a much smaller concentration range (0.001–0.01 μM) than for maximal calcium transport (0.001–0.1 μM) or [^{3}H]inositol release (0.001–10 μM). At 0.01 μM-5-hydroxytryptamine, small increases in the rate of [^{3}H]inositol release or calcium transport were associated with maximal rates of fluid secretion (Fig. 5).

The next series of experiments examined the effect of stimulating fluid secretion with cyclic AMP on inositol release and calcium transport. The addition of 10 mM-cyclic AMP together with 10 μM-3-isobutyl-

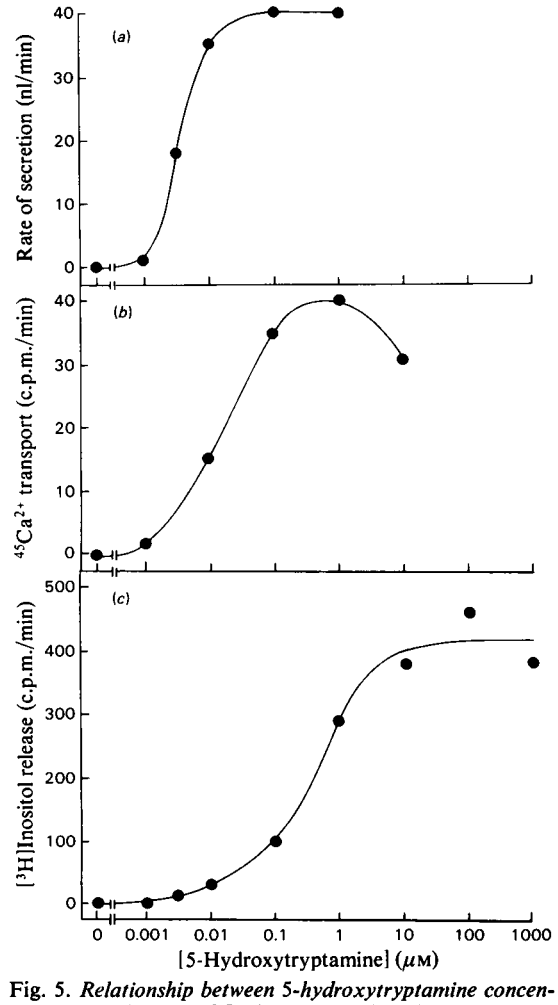

Fig. 5. *Relationship between 5-hydroxytryptamine concentration and the rate of fluid secretion (a), calcium transport (b) and the release of inositol (c)*

For the calcium-transport measurements the protocol was similar to that described in Fig. 4. The values used were those obtained in the 5–10 min period after addition of the different 5-hydroxytryptamine concentrations and the data are plotted as the means for two experiments. Similarly, the protocol for measuring inositol efflux was similar to that described in Fig. 3. The release of inositol to the medium and saliva was measured during the 10–15 min period after the addition of the different 5-hydroxytryptamine concentrations. The data are the means for three separate experiments.

1-methylxanthine gave the same increase in fluid secretion as 0.01 μM-5-hydroxytryptamine plus methylxanthine but had no effect on calcium flux (Fig. 6). There was a transient increase in inositol release to the medium caused by cyclic AMP. Unlike

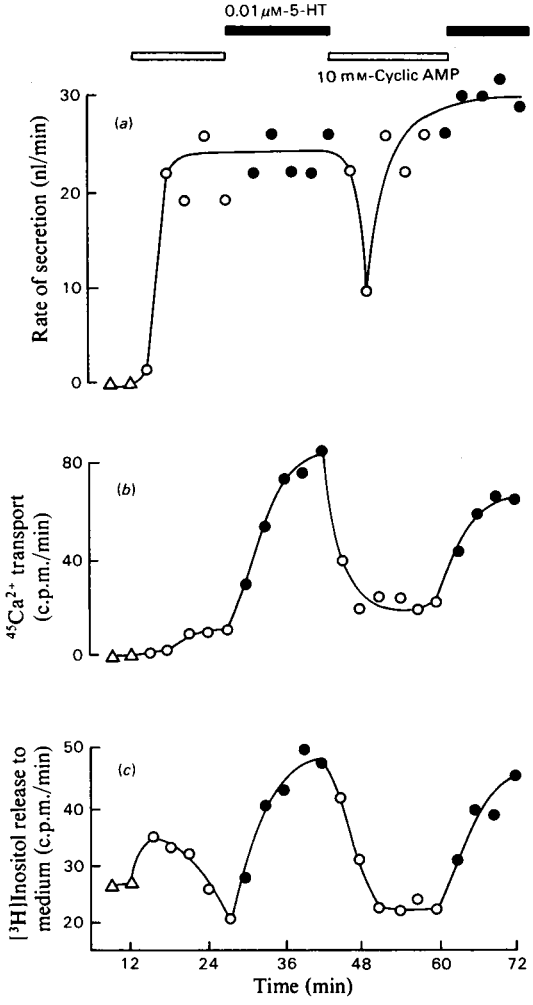

Fig. 6. *Comparison of the effect of cyclic AMP and 5-hydroxytryptamine on fluid secretion (a), transepithelial Ca^{2+} transport (b) and inositol release (c) by salivary glands*

Four salivary glands were incubated for 2h with 20 μM-*myo* [2-^{3}H]inositol and then washed five times over a period of 30min. The amount of label initially present in phosphatidylinositol was 20000c.p.m./gland. The 5-hydroxytryptamine (0.01 μM) and cyclic AMP (10mM) solutions also contained 0.01mM-3-isobutyl-1-methylxanthine and were added alternately over the time periods shown by the open bars for cyclic AMP and by the solid bars for 5-hydroxytryptamine (5-HT). △, Control values before the addition of cyclic AMP. Inositol release to the medium is shown in c.p.m./min for each gland, based on measurements every 3min with the addition of fresh medium at each time point. The $^{45}Ca^{2+}$-flux studies were done with a separate set of glands incubated with 44000c.p.m. of $^{45}Ca^{2+}$/μl. The data represent transfer of $^{45}Ca^{2+}$ from the medium to the saliva in c.p.m./min per gland measured over 3min intervals.

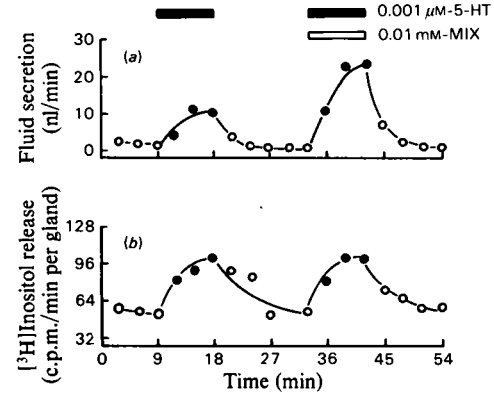

Fig. 7. *Effects of 3-isobutyl-1-methylxanthine on 5-hydroxytryptamine stimulation of fluid secretion (a) and [^{3}H]-inositol release to medium plus saliva (b)*

Four salivary glands were incubated for 2h with 20 μM-*myo*-[2-^{3}H]inositol. The glands were washed five times in fresh medium over a 30min period before the start of the experiment. The solid bars represent the times during which the glands were exposed to 0.001 μM-5-hydroxytryptamine (5-HT) and the open bar when 0.01 mM-3-isobutyl-1-methylxanthine (MIX) was added.

the effect of 5-hydroxytryptamine, inositol release returned to basal values before cyclic AMP was replaced by 5-hydroxytryptamine and was not seen during a second stimulation with cyclic AMP (Fig. 6). In another experimental design, a low rate of fluid secretion caused by 0.001 μM-5-hydroxytryptamine was potentiated by the addition of 10 μM-3-isobutyl-1-methylxanthine, but there was no change in the rate of inositol release (Fig. 7). In a parallel experiment, there was no change in calcium flux when 3-isobutyl-1-methylxanthine was used to increase the secretory response of submaximal doses of 5-hydroxytryptamine (results not shown). These experiments suggest that the hydrolysis of phosphatidylinositol is not some secondary adjunct of the secretory response.

Role of calcium in phosphatidylinositol hydrolysis

To establish that phosphatidylinositol hydrolysis results directly from 5-hydroxytryptamine–receptor interactions, it is necessary to exclude the possibility that the breakdown of phosphatidylinositol is secondary to an increase in the intracellular concentration of calcium due to the increase in calcium entry. If phosphatidylinositol hydrolysis precedes the increase in calcium flux, it should be independent of the external calcium concentration. The insect salivary gland contains an appreciable store of calcium, and it was necessary to ensure that these internal stores were depleted when carrying out

studies in calcium-free buffer. Glands which had been prelabelled with 20 μM-*myo*-[2-^{3}H]inositol were stimulated with 0.1 μM-5-hydroxytryptamine in a calcium-free medium (plus 5 mM-EGTA). As noted previously (Prince *et al.*, 1972; Prince & Berridge, 1973), the secretory response showed a temporary independence of external calcium, but with time the rate of fluid secretion fell to a low value, indicating a fall in the concentration of calcium as the internal reservoirs were progressively depleted (Fig. 8). Despite this fall in the secretory response, the rate of release of inositol remained high, suggesting that phosphatidylinositol hydrolysis was not impaired when the internal calcium concentration was decreased to a value which markedly reduced the secretory response. Removing 5-hydroxytryptamine under calcium-free conditions caused the rate of release of inositol to fall towards the basal value. Replacing calcium had no further effect, but on addition of 5-hydroxytryptamine there was a very large secretory response and the release of inositol returned to the previous values seen during the action of 5-hydroxytryptamine in calcium-free conditions (Fig. 8).

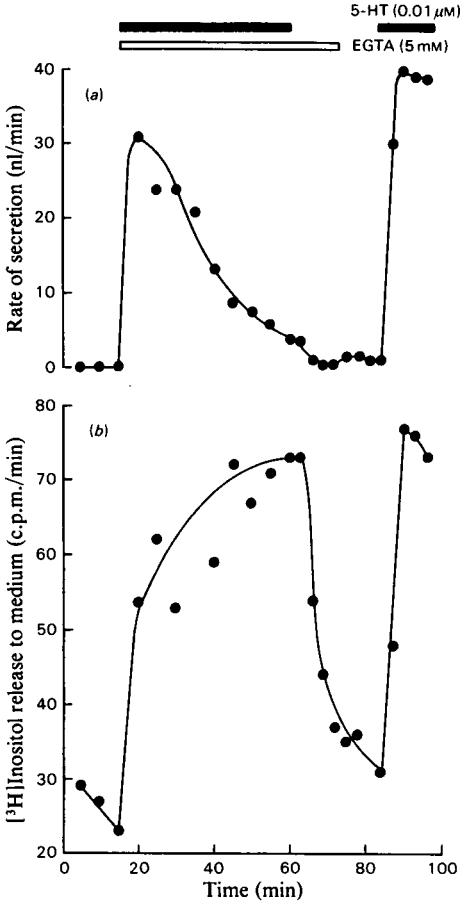

Fig. 8. *Effects of calcium on fluid secretion (a) and phosphatidylinositol breakdown (b)*

Four salivary glands were incubated for 2 h with 20 μM-*myo*-[2-^{3}H]inositol and then washed five times over a 30 min period with unlabelled buffer. The glands were stimulated with 0.01 μM-5-hydroxytryptamine (5-HT; period indicated by the solid bars). During the period indicated by the open bar the buffer containing calcium was replaced with calcium-free buffer containing 5 mM-EGTA. Inositol release to the medium plus saliva is shown in c.p.m./min per gland.

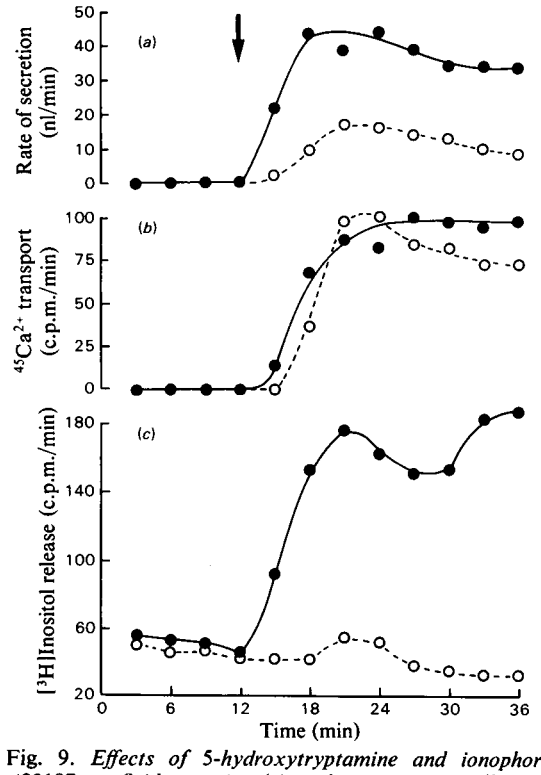

Fig. 9. *Effects of 5-hydroxytryptamine and ionophore A23187 on fluid secretion (a), calcium transport (b) and [^{3}H]inositol release (c)*

Calcium transport was measured by incubating glands in $^{45}Ca^{2+}$ (44000 c.p.m./μl). The rate at which $^{45}Ca^{2+}$ was transferred from the medium to the saliva was measured at 3 min intervals. For the [^{3}H]inositol-release experiment, salivary glands were incubated with 20 μM-*myo*-[2-^{3}H]inositol for 2 h. After removal of the label and washing of the glands, the rate of [^{3}H]inositol efflux to the medium plus saliva was measured at 3 min intervals and 0.05 μM-5-hydroxytryptamine (●) or 1 μM-ionophore A23187 (○) was added after 12 min incubation as indicated by the arrow.

Another way of studying the effect of calcium on phosphatidylinositol hydrolysis is to use the ionophore A23187 to raise the internal concentration of calcium, thus by-passing the 5-hydroxytryptamine receptor. For comparison, concentrations of 5-hydroxytryptamine and ionophore A23187 were selected to produce approximately equal rates of calcium flux across the gland (Fig. 9). At 1 μM, ionophore A23187 produced approximately the same flux of calcium as did 0.05 μM-5-hydroxytryptamine. Despite these similar effects on calcium flux, there was a marked discrepancy in the release of [^{3}H]inositol (Fig. 9). 5-Hydroxytryptamine produced a typical response, whereas ionophore A23187 had almost no effect on the release of label.

Discussion

The phospholipid composition of blowfly salivary glands resembles that of the glands from housefly larvae (Bridges & Price, 1970). Phosphatidylethanolamine (30%) and phosphatidylcholine (21%) are the major phospholipids, with phosphatidylinositol contributing about 8% to the total. When incubated in a relatively simple Ringer solution, [^{32}P]P_i was taken up by all the phospholipids, but a disproportionate amount appeared in phosphatidylglycerol, which probably reflects a shortage of inositol and other precursors for the synthesis of phospholipids. By increasing the inositol concentration it was possible to decrease the amount of label in phosphatidylglycerol, with a corresponding increase in phosphatidylinositol. A similar phenomenon has been described in isolated rat pancreatic islets, where the addition of inositol suppressed the amount of label in CDP-diacylglycerol and phosphatidylglycerol, whereas labelling of phosphatidylinositol was enhanced (Freinkel *et al.*, 1975).

When salivary glands were stimulated with 1 μM-5-hydroxytryptamine for 2h, most of the label that had been incorporated into phosphatidylinositol was released from the cell, whereas there was little or no decrease in the amount of label in the other major phospholipids (Table 2). There was also a marked decrease in the label present in di- and tri-phosphoinositides, but they are present in rather low amounts in comparison with phosphatidylinositol. One reason why we could see a loss of over three-quarters of the newly labelled phosphatidylinositol during 5-hydroxytryptamine stimulation was that synthesis of phospholipids was concurrently inhibited by 5-hydroxytryptamine (Berridge & Fain, 1979). This meant that there was less recycling of the labelled [^{32}P]P_i and [^{3}H]inositol released during 5-hydroxytryptamine-induced breakdown of phosphatidylinositol, and much of this was lost from the cell. This is in contrast with the situation in many mammalian tissues, where the breakdown of phosphatidylinositol by hormones is accompanied by a marked increase in the synthesis of phosphatidylinositol. Nevertheless, this increased rate of resynthesis often does not match the rate of breakdown, thus resulting in a fall in the phosphatidylinositol content of the cell during hormonal stimulation (Michell *et al.*, 1977*a*).

An increased turnover of phosphatidylinositol has been observed during amylase secretion by rat pancreas slices (Hokin & Hokin, 1954), ion transport in the avian salt gland (Hokin & Hokin, 1960) and protein synthesis in the pancreas (Hokin, 1968; Hokin-Neaverson, 1977). Michell (1975) has suggested that the hydrolysis of phosphatidylinositol may be related to the opening of calcium 'gates'. This hypothesis is based on circumstantial evidence that phosphatidylinositol breakdown is associated with stimuli that are also thought to increase the entry of calcium. It seemed important, therefore, to establish whether an increase in calcium permeability is secondary to the hydrolysis of phosphatidylinositol. The blowfly salivary gland appears to be a suitable preparation for such a study, since it was possible continuously to monitor both parameters under identical conditions.

The uptake of $^{45}Ca^{2+}$ into cells has been used extensively as an indicator of membrane permeability to calcium, but this technique is open to criticism and can lead to misleading results (Berridge, 1975). It is also a discontinuous technique and large numbers of samples must be taken in order to establish small changes in calcium permeability. A technique has been developed for salivary glands whereby it is possible continuously to monitor calcium permeability (Berridge & Lipke, 1979). It is important to remember that this technique depends on measuring the net transport of calcium across the gland, which is a two-stage process involving the entry of calcium across the basal membrane followed by its extrusion into the saliva across the apical membrane. Since the entry of calcium across the basal membrane is the rate-limiting step in this transepithelial flux of calcium, it is possible to monitor this permeability continuously by measuring the transport of calcium from the medium into the saliva.

The hydrolysis of [^{3}H]phosphatidylinositol was also followed continuously by measuring the rate at which labelled inositol was released from glands that had been prelabelled with *myo*-[2-^{3}H]inositol. This technique was used previously by Clements & Rhoten (1976) to study phosphatidylinositol metabolism in isolated rat islet cells. Glucose stimulated a marked increase in the efflux of inositol from prelabelled islet cells, with a time course similar to that for the release of insulin. However, the release of inositol was phasic and returned to the control value within 10min, despite the continuous presence of glucose and release of insulin (Clements & Rhoten,

1976). The label released from these islets may have come from inositol intermediates other than phosphatidylinositol because, after the 1 h labelling period, only 15% of the label was in phospholipid, the remainder being water-soluble. However, in the insect salivary gland more than 94% of the label present in the gland was phosphatidylinositol, which may have accounted for the observation that inositol release was not phasic, as in the islet cells, but remained high during continuous stimulation (Figs. 2 and 3). The validity of using inositol release as a continuous assay for phosphatidylinositol hydrolysis was substantiated by studying the cellular concentration of inositol and related intermediates of phosphatidylinositol metabolism. These water-soluble components could not function as the sole source of the [^{3}H]inositol released from the gland, because the concentrations are far too low to maintain the steady release of [^{3}H]inositol observed during continuous stimulation. For example, even in the absence of 5-hydroxytryptamine, the gland released approx. 80% of its intracellular pool of inositol every minute. Therefore, for these internal pools to remain constant and to rise during 5-hydroxytryptamine stimulation (Table 3), it is necessary to assume that they are continuously being replenished by the hydrolysis of phosphatidylinositol.

An increased release of inositol could reflect an accumulation of intracellular inositol resulting from an inhibition of phospholipid synthesis. However, this possibility can be excluded by the finding that, although cyclic AMP or the ionophore A23187 inhibited the synthesis of phosphatidylinositol (Berridge & Fain, 1979), they were not able to stimulate the release of labelled inositol. The release of inositol from the glands appears to be a reliable indicator of the rate of phosphatidylinositol hydrolysis.

An important point to emerge from this study was that both calcium flux and inositol release continued to increase at concentrations of 5-hydroxytryptamine that were far in excess of those necessary for a maximal secretory response. A similar phenomenon has been described in the pancreas, where the extrusion of enzyme reaches a maximum at 0.1 μM-acetylcholine, whereas the hydrolysis of phosphatidylinositol continued to increase up to a concentration of 100 μM (Hokin-Neaverson, 1977). If phosphatidylinositol hydrolysis is related to receptor activation, then such an effect is entirely consistent with the existence of spare receptors. Michell *et al.* (1976) have shown that over a wide range of acetylcholine concentrations there is a close correspondence between the occupation of cholinergic receptors and the hydrolysis of phosphatidylinositol. For hormones such as glucagon and the catecholamines, which act through cyclic AMP, maximal cellular effects can be elicited at concentrations where only 0.01–1.0% of the available receptors are occupied (Levitzki, 1976). At these low hormone concentrations, the increase in cyclic AMP is barely detectable. Further increases in hormone concentration will result in the occupation of more and more of the spare receptors with large increases in the formation of cyclic AMP. A similar phenomenon has been observed in the present study in that maximal rates of fluid secretion were observed with relatively small changes in inositol release and calcium transport (Fig. 6). The fact that inositol release continued to increase over a wide 5-hydroxytryptamine concentration range is certainly consistent with the idea that phosphatidylinositol hydrolysis is linked to the number of occupied receptors.

If the hydrolysis of phosphatidylinositol is related to receptor activation, it should be possible to stimulate fluid secretion without phosphatidylinositol hydrolysis by by-passing the 5-hydroxytryptamine receptor. The studies with cyclic AMP showed that it was possible to obtain rates of fluid secretion that were comparable with those obtained with low doses of 5-hydroxytryptamine but with little or no change in phosphatidylinositol hydrolysis. Similarly, by using the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine, it was possible to enhance greatly the rate of fluid secretion without any change in phosphatidylinositol hydrolysis. In all the cases where fluid secretion was enhanced without activating the 5-hydroxytryptamine receptor, the absence of a phosphatidylinositol response was always associated with little or no change in calcium transport. These studies with cyclic AMP also rule out the possibility that the phosphatidylinositol response is associated with the membrane events responsible for the release of amylase granules by exocytosis. The salivary glands of the blowfly contain large quantities of amylase stored in granules, which are released after stimulation with 5-hydroxytryptamine (Hansen Bay, 1978). Although cyclic AMP and ionophore A23187 can stimulate amylase release, both treatments failed to initiate a significant phosphatidylinositol response, which seemed to occur solely as a consequence of 5-hydroxytryptamine action, suggesting that it may be closely related to the events associated with receptor activation.

Apparently the hydrolysis of phosphatidylinositol is not a secondary consequence of the increase in the intracellular concentration of calcium that occurs when 5-hydroxytryptamine stimulates fluid secretion. When the rate of fluid secretion was used as a physiological indicator of the intracellular concentration of calcium, it was evident that the release of inositol was little affected when the rate of fluid secretion declined to a very low value after the glands had been stimulated with 5-hydroxytryptamine in a calcium-free medium for 45 min (Fig. 8). 5-Hydroxytryptamine stimulates the hydrolysis of phosphatidylinositol under conditions where there is

no movement of calcium across the membrane and where there is no elevation in the intracellular concentration of calcium as judged by salivary secretion. In other systems, external calcium is also not required for the phosphatidylinositol response, as first shown in the cat adrenal medulla (Trifaró, 1969) and later in the rat parotid gland (Jones & Michell, 1975; Oron *et al.*, 1975). Further evidence that the phosphatidylinositol response is not a consequence of an increase in the concentration of calcium was evident from the experiment where ionophore A23187 stimulated a large calcium flux with little or no effect on the release of [^{3}H]inositol. In contrast with the ionophore results, it has not been possible to get a 5-hydroxytryptamine-induced increase in calcium transport without an increase in phosphatidylinositol hydrolysis. These observations are certainly consistent with the view that phosphatidylinositol hydrolysis may be responsible for calcium transport and not vice versa.

The close relationship that exists between [^{3}H]-phosphatidylinositol hydrolysis and calcium transport in this insect salivary gland is consistent with the suggestion that the breakdown of this phospholipid is responsible for gating calcium (Michell, 1975). However, the establishment of such a relationship clearly does not prove the hypothesis, since the key features of the overall mechanism have yet to be established. First, it is not clear how agonists such as 5-hydroxytryptamine activate the enzyme(s) responsible for hydrolysing phosphatidylinositol. Previous studies have shown that one enzyme is likely to be phospholipase C, whose products are 1,2-diacylglycerol, inositol 1:2-cyclic phosphate and inositol 1-phosphate (Michell, 1975). This enzyme seems to function in the insect salivary gland, because during the action of 5-hydroxytryptamine the cellular concentration of labelled inositol 1:2-cyclic phosphate was increased.

Secondly, there is no information on how the hydrolysis of phosphatidylinositol is related to an increased flux of calcium across the membrane. It is possible that 5-hydroxytryptamine increases the availability of phosphatidylinositol for cleavage by phospholipases (Berridge & Fain, 1979). The hydrolysis of phosphatidylinositol could alter the conformation of specific proteins responsible for calcium gating. The ability of 5-hydroxytryptamine to increase calcium permeability is apparently very dependent on the concentration of phosphatidylinositol (Berridge & Fain, 1978). Under conditions where phosphatidylinositol concentration would be expected to decline, there was a marked inactivation of calcium transport. These results suggest a dynamic view of the gating mechanism in which calcium permeability is linked to the rate at which this phospholipid is hydrolysed to diacylglycerol and inositol 1:2-cyclic phosphate.

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