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Inhibition of Phosphatidylinositol Synthesis and the Inactivation of Calcium Entry after Prolonged Exposure of the Blowfly Salivary Gland to 5-Hydroxytryptamine

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The incorporation of [^{32}P]P_i into all salivary-gland phospholipids except phosphatidic acid was inhibited by 5-hydroxytryptamine. The accumulation of [^{32}P]P_i into phosphatidic acid was actually enhanced by 5-hydroxytryptamine. There was an inhibition of labelled inositol incorporation into phosphatidylinositol by 5-hydroxytryptamine, which seems to be mediated by calcium because it was mimicked by the ionophore A23187, but was prevented if glands were stimulated with 5-hydroxytryptamine in the absence of external calcium. Inhibition of synthesis together with stimulation of breakdown will decrease the concentration of phosphatidylinositol, which could account for the inactivation of calcium transport observed at high 5-hydroxytryptamine concentrations. When salivary glands were stimulated with 1 μM-5-hydroxytryptamine, there was a rapid increase in the transfer of $^{45}Ca^{2+}$ from the medium into the saliva, but with time this transport declined to a low value. If the glands were washed free of 5-hydroxytryptamine and incubated in the presence of 2mM-inositol for 1h, the increase in calcium transport caused by 5-hydroxytryptamine was restored. There was little recovery in the absence of inositol. If glands were stimulated with 5-hydroxytryptamine in the absence of external calcium, a condition which prevents the inhibition of phosphatidylinositol synthesis, calcium transport in response to 5-hydroxytryptamine was greater than in glands pre-incubated with 5-hydroxytryptamine in the presence of calcium. The inactivation of calcium transport may result from a decrease in phosphatidylinositol concentration. These results support the hypothesis that the hydrolysis of phosphatidylinositol plays some role in either the opening or closing of calcium 'gates'.

In many mammalian tissues, the addition of hormones or neurotransmitters that stimulate calcium entry results in an increased breakdown of phosphatidylinositol, along with a secondary increase in the resynthesis of this phospholipid (Hawthorne, 1973; Michell, 1975; Michell *et al.*, 1977*a*). The increase in incorporation of [^{32}P]P_i into phosphatidylinositol is not accompanied by a similar increase in the incorporation of labelled glycerol (Michell, 1975). This indicates that there is an increased turnover of the phosphoinositol group, and the diacylglycerol cleaved from phosphatidylinositol is reused by being converted into phosphatidate through the action of diacylglycerol kinase. The synthesis of phosphatidylinositol from phosphatidate involves a reaction with CTP to give CDP-diacylglycerol, which then combines with *myo*-inositol to give phosphatidylinositol and CMP. In many tissues, however, this resynthesis cannot keep pace with the agonist-induced breakdown of phosphatidylinositol, thus resulting in a fall in the tissue content of this phospholipid during hormonal stimulation (Michell *et al.*, 1977*a*). The present study on the insect salivary gland was designed to examine the consequence of such variations in the cellular concentration of phosphatidylinositol on calcium gating.

The salivary glands of the adult blowfly are stimulated to secrete fluid by 5-hydroxytryptamine, calcium being used as a second messenger during stimulus–secretion coupling (Berridge, 1975). In the preceding paper, we have shown that the ability of 5-hydroxytryptamine to increase calcium permeability is closely linked to its ability to stimulate the hydrolysis of phosphatidylinositol (Fain & Berridge, 1979). Unlike the mammalian systems mentioned above, the resynthesis of phosphatidylinositol by this insect salivary gland was not enhanced during hormonal stimulation, but was markedly inhibited, especially at high 5-hydroxytryptamine concentrations. Since 5-hydroxytryptamine stimulates the hydrolysis of phosphatidylinositol while simultaneously decreasing its rate of synthesis, the phosphatidylinositol content of the gland will fall. We have paid particular attention to the consequence of such a decrease in cellular phosphatidylinositol on calcium transport in order to gather further evidence for the proposed role of this phospholipid in calcium gating.

Methods

The experimental procedures used in this study were the same as those described before (Fain & Berridge, 1979). Briefly, for the uptake studies two to four salivary glands were incubated for various times in 50μl of medium containing [^{32}P]P_i or *myo*-[2-^{3}H]inositol. The glands were then washed and the phospholipids were extracted and measured as described previously. The data on inositol incorporation into phosphatidylinositol are expressed as c.p.m./gland or as pmol formed/gland. The latter calculations are based on the specific radioactivity of the *myo*-[2-^{3}H]inositol (5Ci/mmol) in the medium at a concentration of 20μM: 1pmol of phosphatidylinositol formed under these conditions represents 4200 c.p.m.

For the calcium-transport studies, the salivary glands were placed in 50μl drops of medium under liquid paraffin. The cut ends of the isolated glands were ligated with silk threads, which pulled these ends out into the liquid paraffin (Fain & Berridge, 1979). The medium surrounding the closed end of the gland contained $^{45}Ca^{2+}$, and the transepithelial flux of calcium was determined by measuring the rate at which $^{45}Ca^{2+}$ appeared in the saliva (Berridge & Lipke, 1979). The rate of fluid secretion was obtained by measuring the volume of saliva secreted over set intervals. These saliva drops were then transferred by means of Pasteur pipettes to 4ml of Biofluor (New England Nuclear Corp., Boston, MA, U.S.A.) contained in a plastic mini-scintillation vial.

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

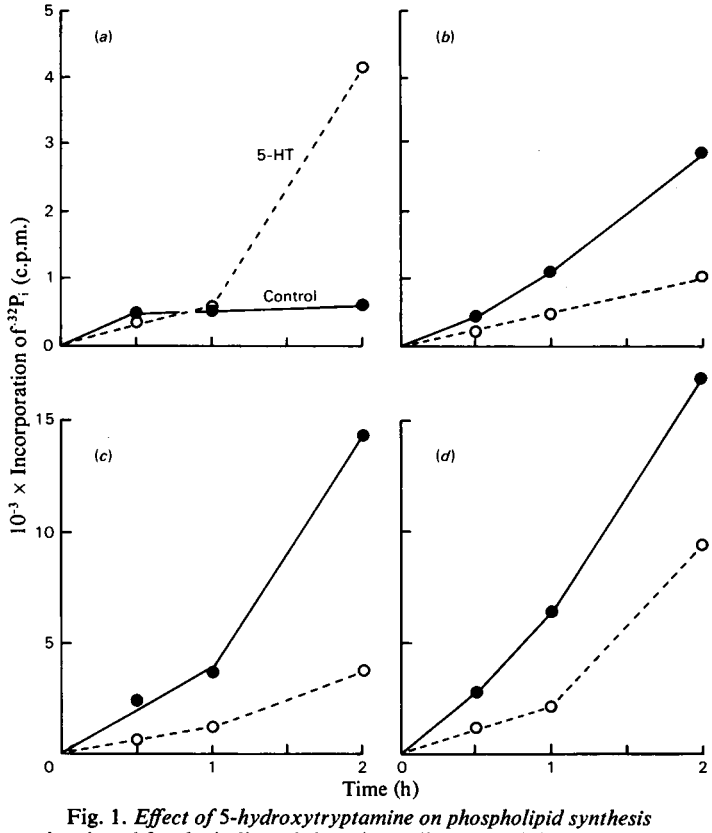

Fig. 1. *Effect of 5-hydroxytryptamine on phospholipid synthesis*
Two salivary glands were incubated for the indicated times in medium containing 5μM-inositol and a tracer amount of [^{32}P]P_i in the absence (●) or presence of 0.1μM-5-hydroxytryptamine (5-HT) (○). Uptake of label into phosphatidate is shown in (*a*), into phosphatidylinositol (*b*), into phosphatidylglycerol (*c*) and into the other phospholipids (phosphatidylcholine, phosphatidylethanolamine and 'X') (*d*).

salivary secretion, Ca^{2+} flux and inositol release. Generally, key experiments were repeated at least twice for the results shown in the Figures but often under slightly different conditions.

Results

Uptake of $[^{32}P]P_i$ and myo-[2-^{3}H]inositol into salivary-gland phospholipids

Salivary glands were incubated with $[^{32}P]P_i$ before separating the individual phospholipids by two-dimensional t.l.c. Most of the label was present in phosphatidylglycerol or the sum of phosphatidylcholine, phosphatidylethanolamine and an unknown phospholipid 'X' (Fig. 1). The pattern of label distribution between the different phospholipids has been described by Fain & Berridge (1979). Phosphatidylinositol accounted for about 10% of the $[^{32}P]P_i$ incorporated into phospholipids under basal conditions. In the presence of 0.1 μM-5-hydroxytryptamine there was an inhibition of $[^{32}P]P_i$ incorporation into all phospholipids except for phosphatidate (Fig. 1). The accumulation of label in phosphatidate was actually enhanced between 1 and 2h of incubation with 5-hydroxytryptamine. The inhibitory effect of 5-hydroxytryptamine is unlikely to result from an inhibition of precursor uptake. At 1h, the uptake of $[^{32}P]P_i$ into water-soluble compounds was unaffected by 0.1 μM-5-hydroxytryptamine and was approx. 50 times that taken up by the phospholipids (results not shown).

A similar inhibition to that of $[^{32}P]P_i$ incorporation into phosphatidylinositol caused by 5-hydroxytryptamine was also seen with respect to *myo*-[2-^{3}H]inositol uptake into this lipid (Fig. 2). Increasing the concentration of inositol from 2 to 2000 μM increased the total amount of phosphatidylinositol formed from 0.5 to 11 pmol/gland over 2h (Fig. 2). The total content of phosphatidylinositol is about 140 pmol/gland (Fain & Berridge, 1979). The inhibitory effect of 5-hydroxytryptamine (0.1 μM) was seen at all concentrations of inositol. The incorporation of [^{3}H]inositol (5 μM) into phosphatidylinositol was linear over a 2h period and was inhibited by 0.1 μM-5-hydroxytryptamine to the same extent at 0.5 or 1h as at 2h (results not shown). Inositol incorporation into phosphatidylinositol was inhibited by 5-hydroxytryptamine concentrations in the range 0.01–1 μM (Figs. 3 and 4).

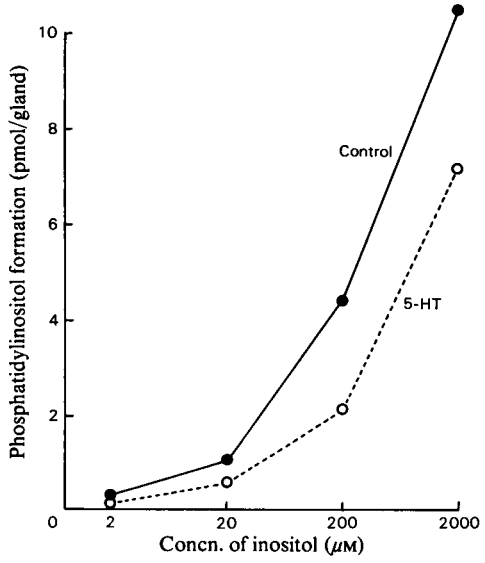

Fig. 2. *Effect of inositol concentration on phosphatidylinositol synthesis*

Two salivary glands were incubated for 2h with the indicated concentrations of *myo*-[2-^{3}H]inositol with (○) or without (●) 0.1 μM-5-hydroxytryptamine.

Role of fluid secretion in inhibition of phosphatidylinositol synthesis by 5-hydroxytryptamine

Cyclic AMP did not stimulate the breakdown of phosphatidylinositol (Fain & Berridge, 1979), but it did inhibit the incorporation of [^{3}H]inositol into this phospholipid. We found that 10 mM-cyclic AMP in the presence of 10 μM-3-isobutyl-1-methylxanthine decreased uptake of 20 μM-[2-^{3}H]inositol into phosphatidylinositol from 34800 ± 3700 to 20000 ± 2900 c.p.m./gland (mean ± S.E.M. for eight experiments). These results suggested that the inhibition of inositol uptake from the medium into phosphatidylinositol was due to a separate effect of 5-hydroxytryptamine unrelated to the increase in phosphatidylinositol breakdown. It is unlikely that some process associated with the mechanism of fluid secretion induced by either 5-hydroxytryptamine or cyclic AMP was responsible for this inhibition of ^{3}H uptake into phosphatidylinositol. As noted previously (Prince & Berridge, 1973), the substitution of chloride in the medium by isethionate (2-hydroxyethanesulphonate) decreased the stimulation of fluid secretion caused by 5-hydroxytryptamine (Fig. 3). Despite this large decrease in fluid secretion, there was no difference in the inhibitory effect of 5-hydroxytryptamine on the uptake of inositol into phosphatidylinositol (Fig. 3).

Role of calcium in the inhibition of phosphatidylinositol synthesis

Possibly the increase in intracellular calcium which occurs during the action of 5-hydroxytryptamine and which can be mimicked by cyclic AMP (Berridge, 1975) might be responsible for the inhibition of phosphatidylinositol synthesis. If this is the case, then increasing intracellular calcium by the

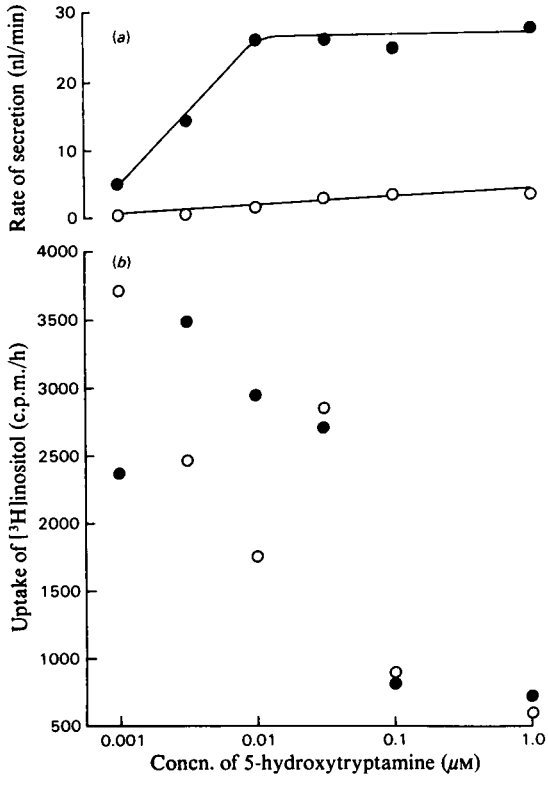

Fig. 3. *Effect of replacing chloride with isethionate on 5-hydroxytryptamine inhibition of inositol uptake*
Two salivary glands were incubated for 2h in the presence of 2μM-*myo*-[2-^{3}H]inositol in regular buffer containing Cl^- (●) or in Cl^--free buffer (○) in which NaCl was replaced by sodium isethionate. Salivary secretion over the first 30min of the 2h incubation is shown in (*a*) and inositol uptake into phosphatidylinositol over 2h in (*b*).

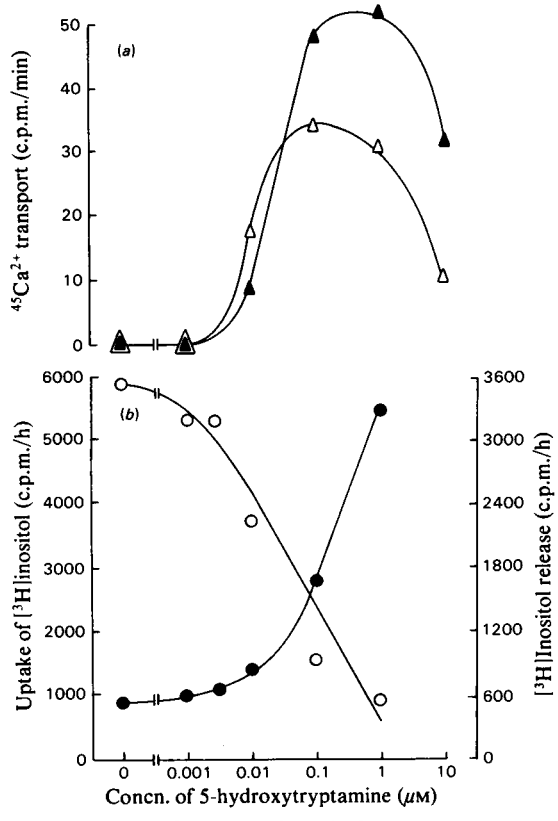

Fig. 4. *Effect of varying the 5-hydroxytryptamine concentration on calcium transport (a) and inositol uptake and release (b)*
(*a*) Calcium transport was determined by incubating glands in a medium containing $^{45}Ca^{2+}$ (7000c.p.m./μl). The rate at which label appeared in the saliva was measured at both 10 (▲) and 60 (△) min after adding different concentrations of 5-hydroxytryptamine. (*b*) For the inositol-uptake measurements (○), two salivary glands were incubated in the presence of 5μM-*myo*-[2-^{3}H]inositol together with the indicated concentrations of 5-hydroxytryptamine. After 2h, the glands were homogenized and the uptake of label into phospholipids was measured in the usual way. The release of inositol (●) was studied in a different set of glands prelabelled for 2h in 5μM-*myo*-[2-^{3}H]-inositol. After this labelling period, the glands were thoroughly washed and the rate at which label was released to the medium was measured over 2h in the presence of different 5-hydroxytryptamine concentrations. The values are the means from six experiments.

addition of the bivalent-cation ionophore A23187 should inhibit inositol incorporation into phosphatidylinositol. The ionophore increased both fluid secretion and calcium transport across the gland, but does not stimulate phosphatidylinositol breakdown (Fain & Berridge, 1979). The results in Table 1 indicate that ionophore A23187 was able to mimic the inhibitory effect of 5-hydroxytryptamine on inositol incorporation into phosphatidylinositol. A concentration of 5-hydroxytryptamine (0.05μM) was selected which produced the same increase in calcium transport as did 1μM-ionophore A23187 (Fain & Berridge, 1979). The combination of both agents appeared to produce an additive effect in inhibiting [^{3}H]inositol incorporation into lipid.

If the increase in intracellular calcium due to 5-hydroxytryptamine, cyclic AMP and the ionophore is responsible for inhibition of phosphatidylinositol synthesis, then, under conditions in which the hormone cannot increase intracellular calcium, there should be no inhibition of inositol incorporation. Salivary glands were incubated for 1h in calcium-free medium plus hormone to deplete intracellular stores of calcium and then incubated for 30min in the presence

of labelled inositol. The basal incorporation into phosphatidylinositol was the same whether the glands were incubated with or without calcium (Table 2). However, the inhibition of inositol incorporation into phosphatidylinositol caused by 5-hydroxytryptamine was abolished in calcium-free conditions. These results indicate that inhibition of phosphatidylinositol synthesis caused by 5-hydroxytryptamine is unrelated to the breakdown of phosphatidylinositol, which occurs to the same extent in the absence or presence of calcium (Fain & Berridge, 1979).

Inactivation of calcium transport

The inhibition of phosphatidylinositol synthesis and the stimulation of its breakdown showed similar sensitivities to 5-hydroxytryptamine over 0.001–1 μM concentration (Fig. 4). In the presence of 1 μM-5-hydroxytryptamine, the release of labelled inositol over 2h represents a net loss of 80% of the labelled phosphatidylinositol present at the beginning (Fain & Berridge, 1979). The loss of this much-labelled phosphatidylinositol coupled with the failure of resynthesis should result in a fall in the concentration of phosphatidylinositol. If the breakdown of phosphatidylinositol is required for the gating of calcium, there should be a decreased calcium flux after prolonged incubation with 5-hydroxytryptamine. This turned out to be the case, since the rate at which radioactive calcium appeared in saliva, measured 60min after the addition of 0.1–10 μM-5-hydroxytryptamine, was considerably less than the rate measured 10min after the addition of 5-hydroxytryptamine (Fig. 4). With a low concentration of 5-hydroxytryptamine (0.01 μM), which does not cause much breakdown of phosphatidylinositol, the rate of appearance of $^{45}Ca^{2+}$ in the saliva was actually greater after 60min than at 10min.

The flux of $^{45}Ca^{2+}$ from the medium to the saliva is a two-stage process involving passive entry across the outer surface, which is the rate-limiting step, followed by active transport into the saliva by pumps on the luminal membrane. While in transit across the gland the label can be diluted by uptake into internal calcium pools, especially when the rate of calcium entry is low (Berridge & Lipke, 1979). When stimulated with 0.01 μM-5-hydroxytryptamine, the lower rates of transport at 10min in Fig. 4 may reflect such a dilution of label in the cytoplasmic compartment. As these internal calcium pools equilibrate with $^{45}Ca^{2+}$, the amount of label appearing in the saliva will gradually increase and more closely reflect the rate of calcium entry from the outside medium.

Prior exposure of glands to high concentrations of 5-hydroxytryptamine (10 μM), which inactivate calcium transport, markedly suppressed the subsequent stimulatory effect of low 5-hydroxytryptamine concentrations on calcium transport. Glands were incubated for 90min in the presence of 10 μM-5-hydroxytryptamine and were then shifted into 0.005 μM-5-hydroxytryptamine (Fig. 5). Fluid secretion was much slower in glands previously exposed to 5-hydroxytryptamine, and a similar effect was

Table 1. *Comparison of the effects of ionophore A23187 and 5-hydroxytryptamine on the uptake of inositol into phosphatidylinositol*

Three salivary glands were incubated for 1h in *myo*-[2-^{3}H]inositol (20 μM) in the presence of 1 μM-ionophore A23187 or 0.05 μM-5-hydroxytryptamine or in a combination of both. At the end of the incubation, the glands were washed five times with control medium before being homogenized in 200 μl of chloroform/methanol (2:1, v/v). The total homogenate was counted for radioactivity and the uptake of label is expressed in c.p.m./gland as the mean ± S.E.M. of nine replicates.

Additions	Inositol uptake into phosphatidylinositol (c.p.m./gland)
None	15990 ± 1610
Ionophore A23187 (1 μM)	7500 ± 1030
5-Hydroxytryptamine (0.05 μM)	5510 ± 480
5-Hydroxytryptamine + ionophore A23187	3440 ± 700

Table 2. *Effect of calcium on the uptake of inositol into phosphatidylinositol*

Three salivary glands were stimulated for 1h with 0.1 μM-5-hydroxytryptamine either in the presence of 2mM-Ca^{2+} or in a calcium-free buffer containing 5mM-EGTA. The glands were then incubated with *myo*-[2-^{3}H]inositol (20 μM) for 1h in the presence or absence of 1 μM-5-hydroxytryptamine and also in the presence or absence of calcium. At the end of the incubation, the glands were washed five times with control medium before being homogenized in 200 μl of chloroform/methanol (2:1, v/v). The total homogenate was counted for radioactivity and the uptake of label is expressed as c.p.m./gland as the mean ± S.E.M. of six replicates.

Condition	5-Hydroxytryptamine concentration (μM)	Inositol uptake into phosphatidylinositol (c.p.m./gland)
Regular buffer with Ca^{2+}	0	17500 ± 1880
Regular buffer with Ca^{2+}	1	2590 ± 440
Ca^{2+}-free buffer with EGTA	0	15380 ± 1390
Ca^{2+}-free buffer with EGTA	1	15240 ± 2140

seen for calcium transport. These results show that loss of responsiveness occurs with respect to both fluid secretion and calcium transport under appropriate conditions.

Inactivation of calcium transport in calcium-free conditions

The importance of extracellular calcium for 5-hydroxytryptamine inactivation of calcium transport was examined. In cells exposed to 5-hydroxytryptamine in calcium-free media, there is normal breakdown of phosphatidylinositol (Fain & Berridge, 1979), but no inhibition of resynthesis (Table 2). Under these calcium-free conditions, the depletion of phosphatidylinositol should be less than that which occurs in the presence of calcium when synthesis is blocked (Table 2). Experiments designed to test this prediction would thus provide another way of establishing the relationship between phosphatidylinositol hydrolysis and calcium transport. Therefore we compared the transport capacity of glands previously incubated for 100min with 10μM-5-hydroxytryptamine in either the presence or the absence of calcium (Fig. 6). A third set of glands

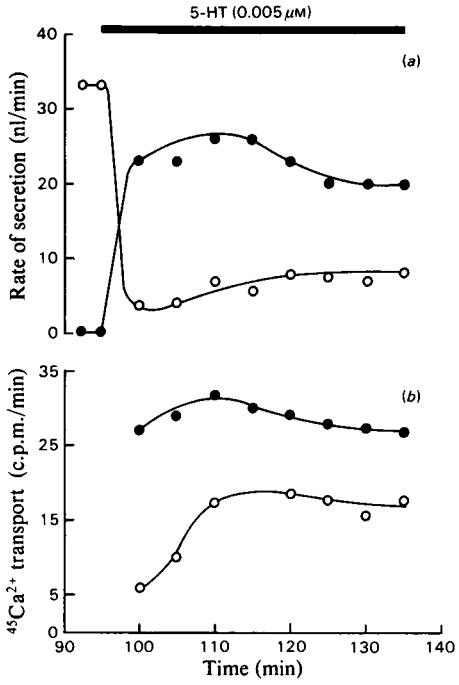

Fig. 5. *Effect of prestimulating salivary glands with a high dose of 5-hydroxytryptamine on their subsequent responsiveness to a sub-maximal concentration of 5-hydroxytryptamine*

Four salivary glands (○) were stimulated for 95min in 10μM-5-hydroxytryptamine (5-HT) and another four glands were incubated in the absence of 5-hydroxytryptamine (●). At 95min both groups were transferred to 0.005μM-5-hydroxytryptamine (shown by the solid bar). Salivary secretion (*a*) and $^{45}Ca^{2+}$ transport (*b*) were measured over the period from 90 to 135min of incubation.

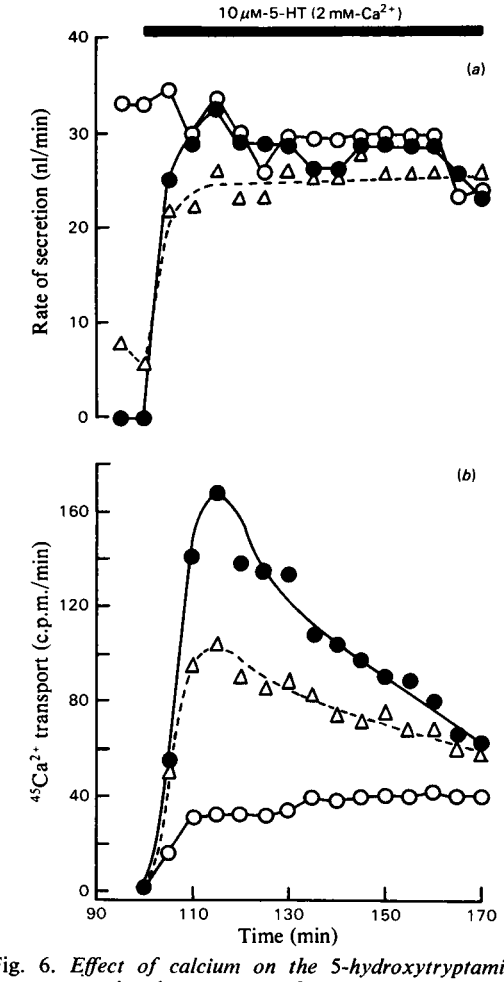

Fig. 6. *Effect of calcium on the 5-hydroxytryptamine-induced inactivation of calcium entry*

Four salivary glands were incubated for 100min in control medium containing 2mM-Ca^{2+} (●; control), or with 1μM-5-hydroxytryptamine (5-HT) plus 2mM-Ca^{2+} (○), or 1μM-5-hydroxytryptamine in a calcium-free medium containing 5mM-EGTA (△). At the end of this 100min stimulation with 1μM-5-hydroxytryptamine, the rate of fluid secretion in the calcium-free conditions had fallen to a low value in comparison with the secretory rate in the presence of calcium (*a*). At 100min all three groups were stimulated with 10μM-5-hydroxytryptamine (containing 2mM-Ca^{2+}) in the presence of $^{45}Ca^{2+}$ (23000c.p.m./μl) to measure the rate of calcium transport (*b*).

were not stimulated for the first 100min and served as controls. After this initial incubation, all three groups were stimulated with 10μM-5-hydroxytryptamine (2mM-calcium) in a medium containing $^{45}Ca^{2+}$ to measure the rate of calcium transport. In the glands previously stimulated with 5-hydroxytryptamine in a calcium-free medium, the rate of calcium transport was much larger compared with that for glands previously stimulated in the presence of calcium (Fig. 6). Stimulation of glands in a calcium-free medium does not afford complete protection, because the response was somewhat less than that obtained in the control glands which had not been exposed to 5-hydroxytryptamine.

The time course of the inactivation of calcium transport is illustrated by the response of the control glands in Fig. 6. During continuous stimulation with 10μM-5-hydroxytryptamine in the presence of 2mM-calcium, the rate of calcium transport rose to a peak and then gradually declined towards the low value found in the glands that had been stimulated under these same conditions from the very beginning of the experiment.

Recovery of calcium transport

Salivary glands were first incubated, with or without 2mM-inositol, for 2h in the presence of 10μM-5-hydroxytryptamine to inactivate calcium transport (Fig. 7). The glands stimulated in the presence of 2mM-inositol showed less inactivation of calcium transport than those which lacked this precursor. 5-Hydroxytryptamine was then removed and the glands were allowed to recover for 1h, after which they were again stimulated with 5-hydroxytryptamine to measure the rate of calcium transport. In the glands allowed to recover in the presence of 2mM-inositol, there was a much greater increase in calcium transport in response to 10μM-5-hydroxy-

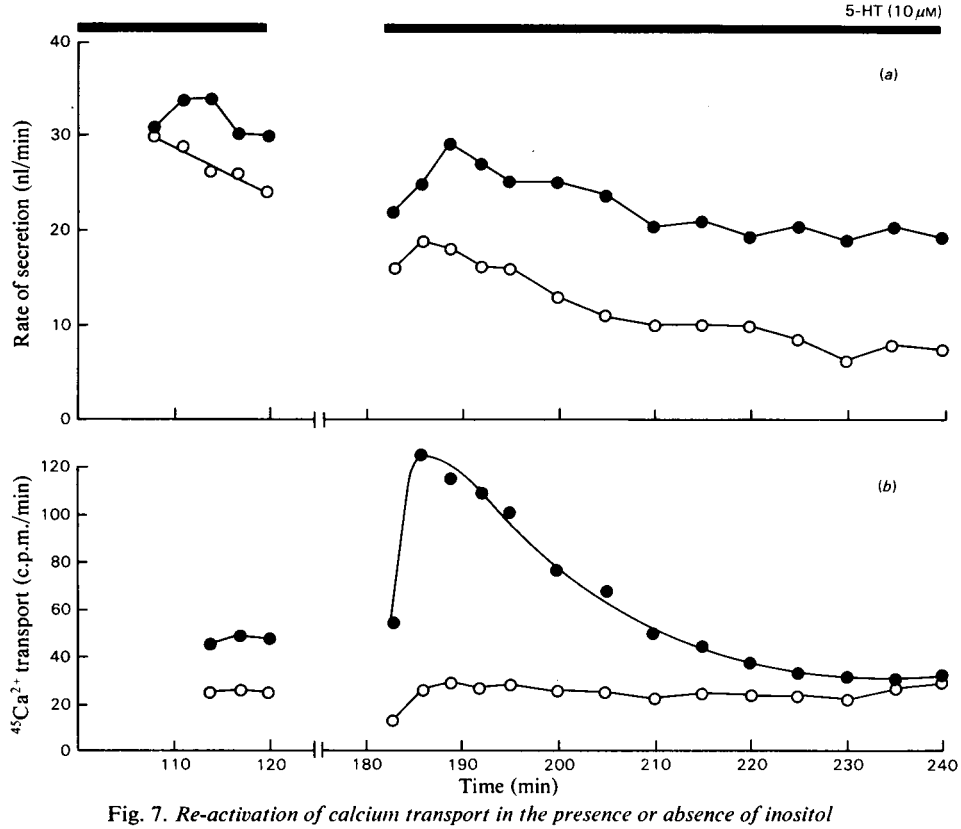

Fig. 7. *Re-activation of calcium transport in the presence or absence of inositol*
Four salivary glands were stimulated for 120min with 10μM-5-hydroxytryptamine (5-HT) in the presence (●) or absence (○) of 2mM-inositol. After removal of 5-hydroxytryptamine, the glands were incubated in control medium for 1h in the presence or absence of 2mM-inositol before being restimulated with 10μM-5-hydroxytryptamine (shown by the solid bar). Salivary secretion (*a*) and $^{45}Ca^{2+}$ transport from the medium to saliva (*b*) were measured between 110 and 120min of incubation and from 183 to 240min of incubation respectively.

tryptamine than in the control glands (Fig. 7). These results suggest that after removal of 5-hydroxytryptamine there is compensatory resynthesis of the phosphatidylinositol, which is needed for a second addition of 5-hydroxytryptamine to gate the entry of calcium.

It was possible to demonstrate a compensatory resynthesis of phosphatidylinositol by comparing the uptake of label into control glands with uptake into glands recovering from a prolonged stimulation with 5-hydroxytryptamine. In glands incubated for 2h with 1 μM-5-hydroxytryptamine before incubation with labelled phosphate or inositol there was a much greater incorporation of label into phosphatidylinositol than occurred in the unstimulated controls (Table 3). There was also an enhanced incorporation of [^{32}P]P_i into all phospholipids, but phosphatidylinositol formation accounted for a much greater fraction of total phospholipid formation than was seen in the absence of inositol (Fain & Berridge, 1979).

In a separate experiment glands incubated for 2h with 10 μM-5-hydroxytryptamine and then incubated for 1h with 2mM-*myo*-[2-^{3}H]inositol formed 14.6 $\pm$ 1.5pmol (mean $\pm$ S.E.M. for six determinations) of phosphatidylinositol during the recovery period compared with 4.1 $\pm$ 0.8pmol in control glands that had not been pre-stimulated with 5-hydroxytryptamine.

Discussion

5-Hydroxytryptamine has a profound effect on phospholipid metabolism in the insect salivary gland. In the preceding paper (Fain & Berridge, 1979) we showed that 5-hydroxytryptamine stimulated the hydrolysis of labelled phosphatidylinositol, but had negligible effects on the remaining phospholipids. In addition to this stimulatory effect on phosphatidylinositol breakdown, high 5-hydroxytryptamine concentrations exerted a more general inhibitory effect on the synthesis of all the major phospholipids except for phosphatidic acid. The way in which 5-hydroxytryptamine inhibits the uptake of label into phospholipids is uncertain. The decreased uptake into phosphatidylinositol cannot be explained by a decrease in the specific radioactivity of [^{3}H]inositol due to the release of non-radioactive inositol liberated by the 5-hydroxytryptamine-induced breakdown of phosphatidylinositol. Cyclic AMP or ionophore A23187 has no effect on the breakdown of phosphatidylinositol, yet they both inhibit the incorporation of label into this phospholipid. Calcium seems to play an important role, because there was no inhibition of synthesis when calcium was removed from the bathing medium. Furthermore, the inhibitory effect of 5-hydroxytryptamine was mimicked by the bivalent-ion ionophore A23187. Therefore the decreased uptake of label into phospholipids seems to reflect a general inhibitory effect of calcium on phospholipid synthesis. The insect salivary gland is unusual with respect to this inhibitory effect because in most other systems hormones or neurotransmitters stimulate the incorporation of [^{32}P]P_i into phosphatidylinositol as well as phosphatidic acid (Michell *et al.*, 1977*a*,*b*). However, these agents also markedly increase the breakdown of phosphatidylinositol. On balance, however, the increase in synthesis fails to keep pace with hydrolysis, resulting in a net fall in the tissue concentration of phosphatidylinositol (Michell *et al.*, 1977*a*,*b*). There probably is a similar decline in the phosphatidylinositol concentration within the insect salivary gland, because 5-hydroxytryptamine not only stimulates the breakdown of this phospholipid but also

Table 3. *Effect of prior incubation with 5-hydroxytryptamine on subsequent uptake of* [^{3}*H*]*inositol and* [^{32}*P*]P_i *into phospholipids*
Salivary glands were incubated for 2h in 1 μM-5-hydroxytryptamine. The glands were extensively washed and then incubated for 30min with 20 μM-*myo*-[2-^{3}H]inositol and [^{32}P]P_i (tracer amount). Groups of two glands were incubated for a further 3h in the presence of unlabelled 1mM-*myo*-inositol and then washed for determination of phospholipid labelling. Control glands were not exposed to 5-hydroxytryptamine during the first 2h.

	Incorporation (c.p.m.)				
	Phosphatidate	Phosphatidylinositol	Phosphatidylglycerol	Phosphatidylcholine	Phosphatidylethanolamine
[^{32}P]P_i incorporation					
Control	1265	10600	4070	5250	2660
Exposed to 5-hydroxytryptamine before labelling	2690	36000	11060	9420	5750
[^{3}H]Inositol incorporation					
Control	90	2300	20	10	10
Exposed to 5-hydroxytryptamine before labelling	90	6400	10	10	10

seems to inhibit its resynthesis. Such a decline in the amount of phosphatidylinositol raises some important questions about the proposed role of this phospholipid in calcium gating.

Michell *et al.* (1977*a*,*b*) suggested that the specific hydrolysis of phosphatidylinositol, which occurs during the activation of many cells by a wide range of hormones or neurotransmitters, plays some role in increasing calcium permeability. In accordance with this hypothesis, we have found a close relationship between the hydrolysis of phosphatidylinositol and the flux of calcium across the insect salivary gland (Fain & Berridge, 1979). If phosphatidylinositol hydrolysis does play an important role in coupling receptor activation to changes in calcium permeability, a decrease in the amount of phosphatidylinositol should result in a change in calcium gating. Indeed, the rate of calcium transport was decreased after prolonged stimulation with high 5-hydroxytryptamine concentrations. These results suggest that the ability of 5-hydroxytryptamine to stimulate calcium transport is somehow determined by the concentration of phosphatidylinositol, which may play some role in the sequence of events linking receptor activation with a change in calcium permeability.

The precise role of phosphatidylinositol hydrolysis in the sequence of events that leads from receptor activation to the opening of the calcium gate is not known. If phosphatidylinositol does play some direct role, then at least two mechanisms are compatible with the observations reported in this paper. The hydrolysis of phosphatidylinositol induced by 5-hydroxytryptamine may result in or be associated with a phasic opening of the calcium gate (Fig. 8). Before this hypothetical calcium gate can be reopened by 5-hydroxytryptamine the phosphatidylinositol which was lost must be replaced by another molecule of phosphatidylinositol in order for the gate to revert from an inactive (5-hydroxytryptamine-insensitive) state to a 'primed' or hormone-sensitive state. It should be emphasized that there is no direct evidence for a structural association between phosphatidylinositol and the hypothetical calcium gate depicted in Fig. 8. The hypothesis we propose is only that the loss of phosphatidylinositol from critical areas in the plasma membrane results in an inability of 5-hydroxytryptamine to stimulate the entry of calcium.

The second mechanism which could account for our observations is that 5-hydroxytryptamine causes

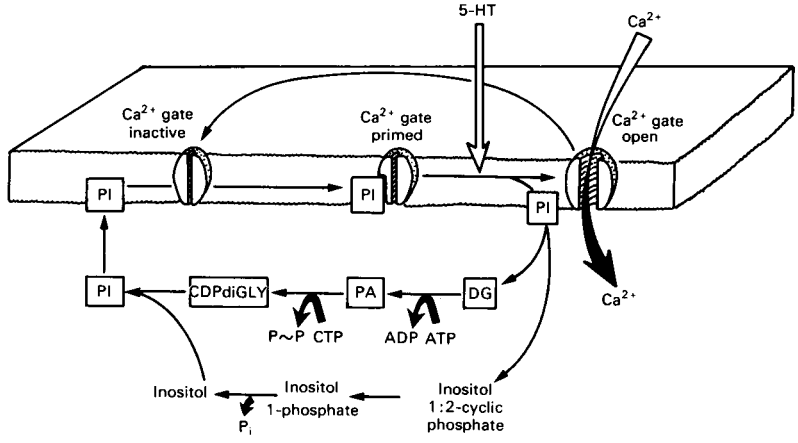

Fig. 8. *Proposed role of phosphatidylinositol hydrolysis in calcium gating*

The hydrolysis of phosphatidylinositol (PI) by 5-hydroxytryptamine (5-HT) is postulated to open specific gates which allow calcium to enter the cell down its electrochemical gradient. One product of the hydrolysis is inositol 1:2-cyclic phosphate, which is converted into inositol 1-phosphate and then into inositol, which can escape from the cell. The other product of the hydrolysis is diacylglycerol (DG). The hydrolysis of phosphatidylinositol may convert the calcium gate into an open state. Alternatively, 5-hydroxytryptamine may open the gate through some conformational change. In the latter mechanism, the calcium gate might be closed by hydrolysis of the phosphatidylinositol, which becomes more accessible to a cytoplasmic phospholipase C-type enzyme when the gate is in the open state. In either case, the gate is converted into a primed or hormone-sensitive state by the rebinding of phosphatidylinositol to the plasma membrane either around or to the calcium gate. In order for 5-hydroxytryptamine to maintain high rates of calcium entry, it apparently is necessary to maintain a high concentration of phosphatidylinositol in the membrane by replacing that which is being degraded. Phosphatidic acid (PA) and CDP-diacylglycerol (CDPdiGLY) are intermediates in the conversion of diacylglycerol into phosphatidylinositol, which is returned to the membrane. Calcium seems to inhibit the synthesis of phosphatidylinositol and the resulting fall in its concentration will slow down the priming of the calcium gate, which thus remains inactive.

a conformational change in the calcium gate which permits entry of calcium. In the open state, phosphatidylinositol bound to the plasma membrane in the vicinity of the calcium gate is more accessible to the hydrolytic action of cytosolic phospholipase C-type enzymes in the presence of hormone. In this mechanism it is the removal of phosphatidylinositol which results in closure of the calcium gate, which then becomes insensitive to 5-hydroxytryptamine unless it is reprimed by binding to another molecule of phosphatidylinositol as described in the first mechanism. The main difference between these two mechanisms therefore depends on whether the hydrolysis of phosphatidylinositol functions as an on or an off signal. Regardless of whether either of the proposed mechanisms is correct, the inactivation of calcium entry seems to result from a decrease in the content of phosphatidylinositol in the gland.

Inactivation of calcium entry is a phenomenon common to many cells which use the influx of external calcium as a signalling device. The physiological importance of this inactivation of calcium entry may be as a safety device to prevent the cell from being swamped with calcium, especially at high hormone titres. In the insect salivary gland, calcium may limit its own entry by decreasing phosphatidylinositol through its ability to inhibit phospholipid synthesis.

Inactivation of calcium entry has also been described in mast cells (Foreman & Garland, 1974), lymphocytes (Freedman *et al.*, 1975) and aortic endothelial cells (D'Amore & Shepro, 1977), all of which probably possess agonist-dependent calcium channels. Inactivation of calcium entry has also been described in those systems, such as squid axon (Baker *et al.*, 1973), insect neurohaemal organ (Maddrell & Gee, 1974), the adrenal medulla (Baker & Rink, 1975) and the neurohypophysis (Nordmann, 1976), which possess voltage-dependent calcium channels. Since the depolarization of ileal smooth muscle with high K^+ increases the incorporation of [^{32}P]P_i into phosphatidylinositol (Jafferji & Michell, 1976), it is conceivable that this phospholipid may play a role in either the opening or closing of these voltage-dependent channels. If phosphatidylinositol hydrolysis does play a role in some of these systems, then on the basis of our observations on the insect salivary gland it is reasonable to propose that inactivation may be secondary to a depletion of phosphatidylinositol at key sites in the membrane surrounding the calcium gates. Such a loss of phosphatidylinositol may also explain the so-called heterodesensitization seen in response to hormones which increase calcium entry into cells. Dale (1958) found that the stimulation of contraction in the guinea-pig ileum by histamine was markedly decreased after acetylcholine treatment. Bown *et al.* (1973) confirmed these findings and noted rapid heterodesensitization with high concentrations of these hormones in the ileum.

In those cases where the onset of inactivation is rapid, the depletion of phosphatidylinositol may arise through mechanisms other than an inhibition of resynthesis. Rapid loss of responsiveness could arise from inhibition of the activity of the specific binding proteins, which are thought to transfer phosphatidylinositol rapidly within the cell. The phenomena of patching and capping in lymphocytes suggests yet another mechanism that could result in a rapid depletion of phosphatidylinositol within a localized region of the membrane. The aggregation of receptors and calcium gates into clumps would limit their access to the remaining phosphatidylinositol dispersed throughout the rest of the cell membrane. In lymphocytes, the time course for cap formation (50% capped by 1.5min; Taylor *et al.*, 1971) is remarkably similar to the time course for the inactivation of calcium entry, which is complete within 1 min of addition of concanavalin A (Freedman *et al.*, 1975).

The best evidence for implicating phosphatidylinositol in calcium gating was obtained by studying the re-activation of calcium transport. In the presence of 2mM-inositol, a significant restoration of calcium transport was apparent 1 h after removal of 5-hydroxytryptamine (Fig. 7). Resynthesis of phosphatidylinositol is an essential feature of this recovery process, because when synthesis was blocked by removing inositol the re-activation of calcium transport was abolished (Fig. 7). It was also possible to demonstrate a compensatory resynthesis of phosphatidylinositol in glands that were recovering after being stimulated for long periods in high concentrations of 5-hydroxytryptamine. The ability to manipulate the recovery of calcium transport by addition or removal of inositol strongly suggests a role for phosphatidylinositol in the gating mechanism.

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References

Baker, P. F. & Rink, T. J. (1975) *J. Physiol. (London)* **253**, 593–620

Baker, P. F., Meves, H. & Ridgeway, E. B. (1973) *J. Physiol. (London)* **231**, 527–548

Berridge, M. J. (1975) *Adv. Cyclic Nucleotide Res.* **6**, 1–98

Berridge, M. J. & Lipke, H. (1979) *J. Exp. Biol.* in the press

Bown, F., Graham, J. D. P. & Taha, S. A. (1973) *Eur. J. Pharmacol.* **22**, 64–74

Dale, M. M. (1958) *Br. J. Pharmacol.* **13**, 17–19

D'Amore, R. & Shepro, D. (1977) *J. Cell. Physiol.* **92**, 177–184

Fain, J. N. & Berridge, M. J. (1979) *Biochem. J.* **178**, 45–58
Foreman, J. C. & Garland, L. G. (1974) *J. Physiol. (London)* **239**, 381–391
Freedman, M. H., Raff, M. C. & Gomperts, B. (1975) *Nature (London)* **255**, 378–382
Hawthorne, J. N. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Hawthorne, J. N. & Dawson, R. M. C., eds.), 2nd edn., pp. 423–440, Elsevier, Amsterdam
Jafferji, S. S. & Michell, R. H. (1976) *Biochem, J.* **160**, 163–169
Maddrell, S. H. P. & Gee, J. D. (1974) *J. Exp. Biol.* **61**, 155–171
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Michell, R. H., Jafferji, S. S. & Jones, L. M. (1977*a*) *Adv. Exp. Biol. Med.* **83**, 447–465
Michell, R. H., Jones, L. M. & Jafferji, S. S. (1977*b*) *Biochem. Soc. Trans.* **5**, 77–81
Nordmann, J. J. (1976) *J. Exp. Biol.* **65**, 669–683
Prince, W. T. & Berridge, M. J. (1973) *J. Exp. Biol.* **58**, 367–384
Taylor, R. B., Duffus, P. H., Raff, M. C. & de Petris. S. (1971) *Nature (London) New Biol.* **233**, 225–229