Phosphoinositide synthesis and Ca^{2+} gating in blowfly salivary glands exposed to 5-hydroxytryptamine

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Blowfly salivary glands, previously exposed to $10\text{ }\mu\text{M}$ -5-hydroxytryptamine for 30 min, demonstrated a rapid compensatory resynthesis of [^3H]inositol-labelled phosphatidylinositol 4,5-bisphosphate when allowed to recover in medium containing 3–5 μM -inositol. Phosphatidylinositol 4,5-bisphosphate comprised 70% of the total [^3H]phosphoinositide, and there was a corresponding decrease in the formation of [^3H]phosphatidylinositol. Subsequent addition of 5-hydroxytryptamine produced an equivalent breakdown of the newly synthesized phosphoinositides but little $^{45}\text{Ca}^{2+}$ gating. Increasing the inositol concentration in the medium to 300 μM produced a 14-fold stimulation of phosphatidylinositol synthesis but only a 5-fold increase in phosphatidylinositol 4,5-bisphosphate synthesis. Increasing the inositol concentration in the medium from 3 μM to 300 μM resulted in a progressively greater recovery of the $^{45}\text{Ca}^{2+}$ -gating response. At 300 μM -inositol there was an 85% recovery of $^{45}\text{Ca}^{2+}$ -gating response. These results indicate that conversion of phosphatidylinositol into phosphatidylinositol 4,5-bisphosphate occurs in blowfly salivary glands and is secondary to an initial breakdown of the phosphoinositides. Recovery of Ca^{2+} gating is dependent on the restoration of both phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate to appropriate concentrations.

Phosphoinositide breakdown has been linked to the mechanism by which hormones such as the α_1 -catecholamines elevate cytosolic Ca^{2+} concentrations (Michell, 1975; Fain, 1982). In blowfly salivary glands 5-HT stimulates breakdown of phosphoinositides, and this is associated with an increased influx of extracellular Ca^{2+} (Fain & Berridge, 1979a). Both $^{45}\text{Ca}^{2+}$ -flux studies (Fain & Berridge, 1979a) and electrophysiological studies (Berridge, 1980) have shown that 5-HT increases the plasma-membrane Ca^{2+} permeability, resulting in an elevation in cytosolic Ca^{2+} concentration. Measurement of the transepithelial $^{45}\text{Ca}^{2+}$ flux due to 5-HT treatment provides a valid index of the hormone-sensitive Ca^{2+} -gating response in these glands. Berridge & Fain (1979) demonstrated that

depletion of the hormone-sensitive pool of PtdIns by prolonged incubation with 5-HT rendered the gland refractory to further hormone-stimulated $^{45}\text{Ca}^{2+}$ entry. Hormone-responsiveness was restored when depleted glands were allowed to resynthesize PtdIns (Berridge & Fain, 1979; Fain & Berridge, 1979b). These findings suggest that the maintenance of a critical pool of PtdIns is necessary to allow hormones to gate Ca^{2+} .

Studies on blowfly salivary glands (Berridge, 1983; Litosch *et al.*, 1984), platelets (Billah & Lapetina, 1982) and rat hepatocytes (Michell *et al.*, 1981) have shown that Ca^{2+} -mobilizing hormones also stimulate the rapid loss of labelled PtdIns(4,5) P_2 . Michell *et al.* (1981) and Berridge (1983) have suggested that all loss of PtdIns is solely due to its conversion into PtdIns(4,5) P_2 , whereas Fain *et al.* (1984) proposed that all phosphoinositides, PtdIns, PtdIns4P and PtdIns(4,5) P_2 , are degraded as a consequence of receptor activation. The present studies were designed to determine the effect of prior exposure to 5-HT on resynthesis of all three phosphoinositides and to correlate phosphoinositide synthesis with the hormone-responsive state.

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; 5-HT, 5-hydroxytryptamine.

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Materials and methods

Salivary glands were obtained from adult blowflies (*Calliphora erythrocephala*) raised at 20–25°C on a diet of sucrose, rat carcasses and water. Flies were used 7–14 days after emergence. The medium for gland dissection and incubation consisted of 150mM-NaCl, 10mM-KCl, 2mM-CaCl₂, 2mM-MgCl₂, 2.7mM-malate, 5.4mM-glutamate, 5mM-glucose and 10mM-Tris/HCl buffer, pH 7.4. All experiments were conducted at room temperature (20–25°C).

Isolated salivary glands were incubated with or without a given concentration of 5-HT for the indicated times. Glands were washed free of 5-HT by transferring them to fresh medium (0.25ml) followed by four additional transfers into 1.0ml of fresh medium over a 1 h wash period. Labelling of salivary-gland phospholipids was based on the procedure of Fain & Berridge (1979a). Briefly, isolated salivary glands were incubated with the indicated concentration of [³H]inositol in 0.10ml of incubation medium. Incubation was terminated by homogenizing glands in 1.5ml of chloroform/methanol (2:1, v/v) containing 0.25% HCl. Phases were separated by addition of 0.3ml of water. The chloroform extract was evaporated to dryness *in vacuo* and resuspended in chloroform. Phospholipids were separated by one-dimensional chromatography on 20cm × 20cm silica gel H plates containing 1% potassium oxalate. The solvent consisted of chloroform/methanol/4M-NH₃/water (90:90:10:19, by vol.) (Schacht, 1978). The identity of phospholipids was confirmed with phospholipid standards. The silica-gel areas containing radioactivity were scraped into liquid-scintillation vials. Water (0.3ml) was added to the powder, followed by 5ml of Biofluor (New England Nuclear). All values were corrected for quench in counting efficiency.

[³H]inositol-labelled phosphoinositides were also separated by two-dimensional chromatography with butan-1-ol/acetic acid/water (6:1:1, by vol.) as described by Abdel-Latif *et al.* (1974). Labelled phosphoinositides were detected by fluorography with the use of a surface-spray autoradiography enhancer. The t.l.c. plate was sprayed with a film of EN³HANCE (New England Nuclear) and exposed to Kodak X-Omat film with screen at –70°C for 96h.

Salivary-gland secretion and ⁴⁵Ca²⁺ flux were determined as described previously (Fain & Berridge, 1979a). Salivary glands were initially incubated in medium containing 0.5mM-inositol for 60min. Glands were then transferred to fresh medium for the beginning of the secretion study. Briefly, salivary glands were set up in 100μl of incubation medium under mineral oil. The open

end of the gland was drawn out from the medium and attached to the bottom of the Petri dish. ⁴⁵Ca²⁺ (1 × 10⁶ c.p.m.) was added to the incubation medium, and the glands were allowed to equilibrate with ⁴⁵Ca²⁺ for 30 min before the start of the experiment. The secretory volume was determined by measuring the diameter of the secretory droplet that formed at the open end of the gland. The amount of ⁴⁵Ca²⁺ that appeared in the secretory droplet was determined by counting the radioactivity in the droplet with the use of 5 ml of Biofluor. All additions of 5-HT and inositol were made to the incubation medium.

myo-[2-³H]inositol (10Ci/mmol) was from Amersham/Searle. ⁴⁵Ca²⁺ was from New England Nuclear. Pure phospholipid standards PtdIns(4,5)P₂, PtdIns4P and PtdIns were from Sigma Chemical Co.

Results

Salivary glands incubated in medium containing 5 μM-[³H]inositol incorporated approx. 38% of the lipid label into PtdIns(4,5)P₂, 12% into PtdIns4P and 50% into PtdIns (Table 1). Exposure of glands to 10 μM-5-HT for 2h before the start of the labelling period resulted in a marked redistribution of the incorporated label. PtdIns(4,5)P₂ now accounted for 70% of the lipid label, whereas PtdIns constituted only 16% of the total label. There was no effect of prior exposure to 5-HT on the amount of label present in PtdIns4P or on the total amount of inositol label incorporated between control and 5-HT-pretreated glands under these conditions (Table 1). These results indicate that prior exposure to 5-HT induces a compensatory resynthesis of labelled PtdIns(4,5)P₂ that occurs at the expense of PtdIns.

To ensure that the measured changes in radioactivity were indeed in PtdIns(4,5)P₂, the [³H]-inositol-labelled phospholipids were separated by two-dimensional chromatography by using previously defined procedures (Abdel-Latif *et al.*, 1974; Schacht, 1978; Litosch *et al.*, 1983) and detected by autoradiography. As shown in Fig. 1(a), only three lipids were visibly labelled, and these corresponded to PtdIns(4,5)P₂, PtdIns4P and PtdIns, as identified by co-migration with pure phosphoinositide standards. The effect of prior exposure to 5-HT on the incorporation of label into the phosphoinositides is shown in Fig. 1(b). There was a marked increase in the amount of radioactivity incorporated into PtdIns(4,5)P₂ and a decrease in that incorporated into PtdIns.

A time course for the uptake of [³H]inositol into PtdIns(4,5)P₂ from control and 5-HT-pretreated glands is shown in Fig. 2. The stimulated uptake of label into PtdIns(4,5)P₂ from hormone-pretreated

Table 1. *Effect of Ca^{2+} on 5-HT-stimulated phosphoinositide resynthesis*

Salivary glands were treated with $10\text{ }\mu\text{M}$ -5-HT for 30 min in regular Ca^{2+} buffer (2.0 mM-Ca^{2+}) or Ca^{2+} -free buffer containing $50\text{ }\mu\text{M}$ - or $250\text{ }\mu\text{M}$ -EGTA. Glands were washed free of 5-HT, and then labelled in regular buffer containing $5\text{ }\mu\text{M}$ - $[^3\text{H}]$ inositol for 60 min. Results are the means $\pm$ S.E.M. for six determinations. Four determinations were made with $50\text{ }\mu\text{M}$ -EGTA, and two were made with $250\text{ }\mu\text{M}$ -EGTA. The total radioactivity (c.p.m.) incorporated into each phosphoinositide is shown, with three glands each condition. The percentage change in label incorporation due to 5-HT for each paired determination is indicated in parenthesis: * $P \leq 0.025$; ** $P \leq 0.010$; *** $P \leq 0.001$.

Pretreatment conditions ...	Uptake of $[^3\text{H}]$ inositol over 60 min (c.p.m.)			
	Regular buffer + Ca^{2+}		Ca^{2+} -free + EGTA	
	Control	+ 5-HT	Control	+ 5-HT
Phosphoinositide				
PtdIns(4,5) P_2	4960	9060 (+92 $\pm$ 27%)*	4570	7910 (+75 $\pm$ 16%)*
PtdIns4P	1620	1660 (+7 $\pm$ 9%)	1490	1426 (-16 $\pm$ 5%)
PtdIns	6620	2020 (-63 $\pm$ 8%)*	6880	4500 (-36 $\pm$ 8%)*
Total uptake	13190 $\pm$ 2680	12740 $\pm$ 3230	12940 $\pm$ 3300	13840 $\pm$ 337

Fig. 1. *Autoradiography of salivary-gland $[^3\text{H}]$ inositol-labelled phosphoinositides obtained from control and 5-HT-pretreated glands*

Salivary glands were incubated with or without $10\text{ }\mu\text{M}$ -5-HT for 30 min. Glands were washed and then incubated for 2.5 h with $5\text{ }\mu\text{M}$ - $[^3\text{H}]$ inositol. Glands were homogenized in 1.5 ml of acidified chloroform/methanol (2:1, v/v), and lipids were extracted. The $[^3\text{H}]$ inositol-labelled phosphoinositides were separated by two-dimensional chromatography and detected by using fluorography as described in the Materials and methods section. The total amount of labelled phosphoinositide in control glands (a) was 6000 c.p.m., and in 5-HT-pretreated glands (b) it was 5600 c.p.m.

glands was apparent within 5 min of incubation with label. There was no apparent time lag for the increase in labelling. By 60 min, the amount of labelled PtdIns(4,5) P_2 was approx. 330% of the control value.

Near-maximal effects of 5-HT on the compensatory resynthesis of PtdIns(4,5) P_2 and PtdIns were obtained with $0.1\text{ }\mu\text{M}$ -5-HT (Fig. 3). There was little additional effect of higher 5-HT concentrations.

The maximal length of exposure to $10\text{ }\mu\text{M}$ -5-HT required to induce a compensatory resynthesis of labelled PtdIns(4,5) P_2 was 30 min (Fig. 4). However, some effect of 5-HT could be seen after only a 5 min exposure to $10\text{ }\mu\text{M}$ -5-HT (Fig. 4).

Since one consequence of 5-HT stimulation is to increase the entry of extracellular Ca^{2+} (Fain & Berridge, 1979a), the role of Ca^{2+} in the compensatory resynthesis of PtdIns(4,5) P_2 was examined. The effect of stimulating salivary glands with 5-HT

Fig. 2. Effect of prior exposure to 5-HT on $[^3\text{H}]$ inositol uptake into salivary-gland $\text{PtdIns}(4,5)\text{P}_2$

Salivary glands were incubated with (○) or without (●) $10\ \mu\text{M}$ -5-HT for 2 h. Glands were washed free of 5-HT and then incubated with $5\ \mu\text{M}$ - $[^3\text{H}]$ inositol for the indicated times. Results are the means for three experiments.

Fig. 3. Dose-response curve for 5-HT effects on phosphoinositide resynthesis

Salivary glands were incubated with the indicated concentration of 5-HT for 30 min followed by washing to remove 5-HT. Glands were then incubated for 60 min with $3\ \mu\text{M}$ - $[^3\text{H}]$ inositol. Results are the means for two determinations of PtdIns (○) and $\text{PtdIns}(4,5)\text{P}_2$ (●).

in Ca^{2+} -free buffer containing $50\ \mu\text{M}$ - or $250\ \mu\text{M}$ -EGTA on the compensatory resynthesis of $\text{PtdIns}(4,5)\text{P}_2$ is shown in Table 1. The 5-HT effect was slightly attenuated but still evident in glands that had been pre-exposed to 5-HT in Ca^{2+} -free medium. These data suggest that the compensatory resynthesis of $\text{PtdIns}(4,5)\text{P}_2$ is a consequence of the 5-HT-induced depletion of PtdIns and

Fig. 4. Effect of length of exposure to 5-HT on subsequent synthesis of $\text{PtdIns}(4,5)\text{P}_2$

Salivary glands were incubated with or without $10\ \mu\text{M}$ -5-HT for the indicated times. Glands were washed free of 5-HT and then incubated with $5\ \mu\text{M}$ - $[^3\text{H}]$ inositol for 60 min. Uptake of label into $\text{PtdIns}(4,5)\text{P}_2$ in glands with no prior exposure to 5-HT (zero time) and glands previously exposed to 5-HT for the indicated times is shown.

$\text{PtdIns}(4,5)\text{P}_2$ rather than an effect secondary to entry of extracellular Ca^{2+} .

Synthesis of PtdIns by salivary glands is dependent on the inositol concentration in the medium (Berridge & Fain, 1979). Raising the inositol concentration in the medium from $3\ \mu\text{M}$ to $300\ \mu\text{M}$ increased the synthesis of all phosphoinositides (Table 2). It was observed during these particular studies that salivary glands not previously exposed to 5-HT were forming more labelled $\text{PtdIns}(4,5)\text{P}_2$ than had been earlier observed (Litosch *et al.*, 1984), and thus resembled 5-HT-pretreated glands. Approx. 40% of the incorporated label was present in $\text{PtdIns}(4,5)\text{P}_2$ in control glands incubated in the presence of $3\ \mu\text{M}$ -inositol. This suggested that salivary glands were being exposed to minute amounts of endogenous amines, possibly released from secretory granules that triggered a compensatory resynthesis of $\text{PtdIns}(4,5)\text{P}_2$. Isolation of glands in medium containing $100\ \mu\text{M}$ -gramine, an indoleamine that antagonizes interaction of 5-HT with its receptor, resulted in a decrease in the amount of labelled $\text{PtdIns}(4,5)\text{P}_2$ formed as compared with control glands isolated without gramine (Table 2). In gramine-treated glands, $\text{PtdIns}(4,5)\text{P}_2$ constituted 27% of the total incorporated label, consistent with previous studies (Litosch *et al.*, 1984). These results suggest that salivary glands may synthesize variable amounts of labelled $\text{PtdIns}(4,5)\text{P}_2$, possibly as a consequence of the release of endogenous tryptamines that

Table 2. *Effect of inositol concentration on synthesis of phosphoinositides*

Salivary glands were incubated with 10 μ M-5-HT, 100 μ M-gramine or no addition for 30 min. Treated glands were washed free of agents and then incubated in the presence of 3 μ M-, 30 μ M- or 300 μ M-inositol for 60 min. Results are the means $\pm$ S.E.M. for three experiments. Abbreviation: N.D., not determined.

Phosphoinositide	Treatment	Concn. of inositol	Synthesis (pmol/gland)		
			3 μ M	30 μ M	300 μ M
PtdIns(4,5) P_2	5-HT		0.14 $\pm$ 0.02	0.59 $\pm$ 0.08	0.70 $\pm$ 0.04
PtdIns4P			0.05 $\pm$ 0.01	0.11 $\pm$ 0.02	0.34 $\pm$ 0.02
PtdIns			0.08 $\pm$ 0.01	0.64 $\pm$ 0.21	1.12 $\pm$ 0.20
PtdIns(4,5) P_2	Gramine		0.07 $\pm$ 0.02	0.27 $\pm$ 0.11	0.53 $\pm$ 0.04
PtdIns4P			0.03 $\pm$ 0.01	0.13 $\pm$ 0.05	0.34 $\pm$ 0.03
PtdIns			0.16 $\pm$ 0.03	0.83 $\pm$ 0.26	1.24 $\pm$ 0.30
PtdIns(4,5) P_2	Control		0.11 $\pm$ 0.01	0.37 $\pm$ 0.09	N.D.
PtdIns4P			0.03 $\pm$ 0.01	0.22 $\pm$ 0.04	N.D.
PtdIns			0.13 $\pm$ 0.01	0.65 $\pm$ 0.11	N.D.

trigger a compensatory resynthesis of PtdIns(4,5) P_2 .

In 5-HT-pretreated glands approx. 50% of the inositol lipid formed at 3 μ M-inositol was PtdIns(4,5) P_2 . Increasing the inositol concentration to 30 μ M resulted in a 5-fold increase in total lipid inositol. There was an 8-fold increase in the amount of PtdIns and a 4-fold increase in the amount of PtdIns(4,5) P_2 formed. At 300 μ M-inositol, the total lipid content increased by 8-fold. The major increase was in PtdIns, which increased 14-fold. In contrast, PtdIns(4,5) P_2 increased by only 5-fold from the value obtained at 3 μ M-inositol (Table 2). Control glands (not treated with gramine or 5-HT) also showed a proportionally greater synthesis of labelled phosphatidylinositol as compared with PtdIns(4,5) P_2 with increasing inositol concentrations. In contrast, gramine-treated glands maintained a relatively constant distribution of labelled PtdIns(4,5) P_2 at all inositol concentrations examined (Table 2).

These results suggest that depletion of the labelled pool of PtdIns(4,5) P_2 and PtdIns by 5-HT results in a compensatory resynthesis of PtdIns(4,5) P_2 at the expense of PtdIns at low inositol concentrations in the medium. As the pool of PtdIns(4,5) P_2 is replenished by incubation of glands in medium containing increasing inositol concentrations, PtdIns synthesis begins to predominate and eventually this becomes the major phosphoinositide.

Fain & Berridge (1979b) demonstrated that glands previously exposed to 5-HT were unable to gate $^{45}\text{Ca}^{2+}$ on a second exposure to hormone unless they recovered their PtdIns content. The 5-HT-stimulated Ca^{2+} -gating response from control glands and hormone-treated glands that were allowed to recover in medium containing three different concentrations of inositol is shown in Fig.

5. Isolated salivary glands exhibited a low basal rate of secretion and transepithelial $^{45}\text{Ca}^{2+}$ movement. Addition of 10 μ M-5-HT to glands not previously exposed to hormone stimulated both salivary-gland secretion and $^{45}\text{Ca}^{2+}$ gating (Fig. 5). Both secretion and $^{45}\text{Ca}^{2+}$ flux were linear for the first 15 min of hormone addition. At 30 min there was a slight decline in $^{45}\text{Ca}^{2+}$ gating, which may be related to the depletion of a cellular pool of phosphoinositides involved in Ca^{2+} gating (Fain & Berridge, 1979b). The degree of $^{45}\text{Ca}^{2+}$ gating in pre-exposed glands was clearly dependent on the inositol concentrations in the recovery medium. These results demonstrate that glands that had recovered in 3 μ M-inositol had only 23% of the control response. As the inositol concentration was increased to 30 μ M, approx. 50% of the control response was observed. At 300 μ M, there was no significant difference between pre-exposed glands and glands not previously exposed to 5-HT.

The secretory responses to 5-HT in all groups were similar (Fig. 5), indicating that the altered $^{45}\text{Ca}^{2+}$ flux was independent of changes in secretion. These results indicate that restoration of cellular phosphoinositides to appropriate concentrations is essential to obtain an optimal Ca^{2+} -gating response, and are consistent with previous studies (Fain & Berridge, 1979b) that showed a progressive recovery of the $^{45}\text{Ca}^{2+}$ -gating response dependent on the inositol concentration in the recovery medium.

The effect of 5-HT on phosphoinositide breakdown in glands that had recovered with 3 μ M-inositol was examined. As shown in Table 3, 5-HT stimulated breakdown of approx. 40% of the labelled phosphoinositides. Taken together with the data in Fig. 5, these data indicate that breakdown of phosphoinositides occurs under conditions where there is little Ca^{2+} gating. Further-

Fig. 5. Time course for the effect of 5-HT on $^{45}\text{Ca}^{2+}$ gating and secretion for control and 5-HT-pretreated glands. Control ($\square$) and 5-HT-pretreated glands allowed to recover in medium containing 3 μM - ($\circ$), 30 μM - ($\blacksquare$) or 300 μM - inositol ($\bullet$) were incubated for 30 min in incubation medium containing $^{45}\text{Ca}^{2+}$ to establish a basal rate of $^{45}\text{Ca}^{2+}$ flux and secretion. At the indicated time, 10 μM -5-HT was added to the incubation buffer, and the amount of $^{45}\text{Ca}^{2+}$ gated (a) and salivary secretion (b) were determined over a 30 min incubation period. The basal $^{45}\text{Ca}^{2+}$ gating was 23 ± 5 , 29 ± 9 , 47 ± 19 and 36 ± 15 c.p.m. for control and glands allowed to recover in medium containing 3 μM -, 30 μM - and 300 μM -inositol respectively. In glands previously exposed to 5-HT and allowed to recover in the presence of 3 μM -inositol, the percentage decrease in $^{45}\text{Ca}^{2+}$ gating was 84 ± 7 , 82 ± 5 , 78 ± 6 and 73 ± 6 at 5, 10, 15 and 30 min as the means $\pm$ S.E.M. for the paired differences, and this was significant with $P < 0.001$ at all time points. In glands allowed to recover with 30 μM -inositol, the percentage decrease was 57 ± 16 , 59 ± 14 , 55 ± 15 and 47 ± 16 for the indicated time points, and this was significant with $P < 0.010$, 0.005 , 0.010 and 0.025 respectively. Glands allowed to recover in medium containing 300 μM -inositol had a percentage decrease of 21 ± 27 , 19 ± 23 , 17 ± 6 and 24 ± 13 respectively for these time points, and this was not significantly different from control. The basal rate of secretion was 1 ± 1 , 1 ± 1 , 6 ± 4 and 2 ± 1 nl for control glands and glands allowed to recover in medium containing 3 μM -, 30 μM - and 300 μM -inositol. The secretion at 15 min due to 5-HT in control and glands allowed to recover in medium containing 3 μM -, 30 μM - and 300 μM -inositol was 224 ± 40 , 174 ± 64 , 227 ± 85 and 259 ± 87 nl respectively. The standard error for control values was 18% of the mean whereas in pretreated glands it was 33–38% of the mean. The results are the means for 22 determinations for control glands, six determinations for glands allowed to recover in 3 μM -inositol and eight determinations each for 30 μM - and 300 μM -inositol.

Table 3. Effect of 5-HT on phosphoinositide breakdown in salivary glands previously exposed to 5-HT

Salivary glands treated with 10 μM -5-HT for 30 min were washed free of 5-HT followed by labelling for 60 min with 5 μM -[^3H]inositol. The labelled glands were incubated with or without 10 μM -5-HT for 5 min. Results shown are the means $\pm$ S.E.M. for five experiments, with 15 glands for each condition.

* $P \leq 0.010$; ** $P \leq 0.005$.

Phosphoinositide	Radioactivity in control (c.p.m.)	Change due to 5-HT (%)
PtdIns(4,5) P_2	25575	$-36 \pm 8^*$
PtdIns4P	5480	$-34 \pm 5^{**}$
PtdIns	11210	$-40 \pm 7^{**}$

more, the newly synthesized phosphoinositides are degraded to an equivalent extent by a subsequent exposure to 5-HT.

Discussion

The present studies demonstrate that one effect of prior 5-HT stimulation in blowfly salivary glands is to increase the subsequent resynthesis of PtdIns(4,5) P_2 from PtdIns. This effect was best seen when the inositol concentration in the medium was 5 μM or less. Maximal effects of 5-HT on compensatory resynthesis of PtdIns(4,5) P_2 were obtained after a 30 min incubation with 5-HT in the presence of Ca^{2+} in the medium. Under

these conditions the pool of PtdIns and PtdIns(4,5) P_2 involved in hormone action should have been maximally depleted as a result of stimulation of breakdown and inhibition of synthesis (Fain & Berridge, 1979a; Litosch *et al.*, 1984).

Berridge & Fain (1979) reported an enhanced incorporation of [^{3} H]inositol into PtdIns in 5-HT-pretreated glands as compared with glands not previously exposed to 5-HT. Uptake of label into polyphosphoinositides was not examined. There was an approx. 3-fold increase, over control, in the amount of labelled inositol lipid formed when glands were allowed to recover in medium containing 200–2000 μ M-inositol. These findings contrast with the results of the present study, which demonstrate that the total amount of inositol lipid synthesized at 300 μ M-inositol was similar in control and 5-HT-pretreated glands. Although the reason for the discrepancy is not readily apparent, in the studies by Berridge & Fain (1979) there was also a 3-fold increase in turnover of other phospholipids, including phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol and phosphatidic acid, in pretreated glands. Thus the increased [^{3} H]inositol incorporation into PtdIns may have reflected a generalized increase in lipid turnover. In the present studies the total amount of [^{3} H]inositol lipid synthesized at 3 μ M-, 30 μ M- or 300 μ M-inositol in control and pretreated glands was similar (Table 2). The marked redistribution of [^{3} H]inositol incorporated into the phosphoinositides was only observed at low inositol concentrations (3–30 μ M) in the medium, which were not examined by Berridge & Fain (1979).

It is not clear why depletion of PtdIns and PtdIns(4,5) P_2 should trigger such a marked effect on subsequent PtdIns(4,5) P_2 resynthesis. The nature of the metabolic regulation that favours a preferential resynthesis of the small pool of PtdIns(4,5) P_2 over PtdIns is not known. PtdIns(4,5) P_2 synthesis presumably occurs through a rapid phosphorylation of PtdIns and PtdIns4P by PtdIns kinase and PtdIns4P kinase respectively. The increased formation of labelled PtdIns(4,5) P_2 , however, was associated with a poor $^{45}\text{Ca}^{2+}$ -gating response in glands desensitized by a previous exposure to 5-HT and incubated with 3 μ M-inositol. 5-HT stimulated an equivalent breakdown of PtdIns(4,5) P_2 and PtdIns in these glands, but there was very little $^{45}\text{Ca}^{2+}$ gating in these glands. In contrast, at 300 μ M-inositol there was full recovery of $^{45}\text{Ca}^{2+}$ gating. These results are similar to those published by Berridge & Fain (1979), who demonstrated that recovery of hormone-stimulated Ca^{2+} gating required at least 200 μ M-inositol in the medium. The secretory response that utilizes both cyclic AMP and Ca^{2+} as second messengers was not affected by

pre-exposure to 5-HT, in agreement with the studies by Berridge & Fain (1979) and Fain & Berridge (1979b).

The ability of 5-HT to stimulate an equivalent breakdown of PtdIns and PtdIns(4,5) P_2 under conditions in which there was little Ca^{2+} gating indicates that desensitization is not due to a defect in the ability of 5-HT to interact with its receptor and produce an apparent activation of phospholipase C activity. Rather, the data suggest that desensitization results from depletion of phosphoinositides from a cellular site essential to Ca^{2+} -gating function. Breakdown of a critical amount of phosphoinositides may be necessary to open the hormone-sensitive Ca^{2+} gate. This effect could be related to the formation of phosphoinositide breakdown products that trigger Ca^{2+} mobilization. Breakdown of salivary-gland phosphoinositides results in the formation of diacylglycerol (Litosch *et al.*, 1982) and the corresponding inositol phosphates (Berridge *et al.*, 1983; Litosch *et al.*, 1984). Diacylglycerol or inositol phosphates may act as second messengers to increase entry of extracellular Ca^{2+} . In glands depleted of phosphoinositides the production of these putative messengers would be diminished and there would be a corresponding decrease in the general signal.

Recent studies by Streb *et al.* (1983) have shown that the addition of inositol 1,4,5-trisphosphate to permeabilized pancreatic acinar cells produced a transient efflux of Ca^{2+} from an intracellular site, presumably endoplasmic reticulum. This suggests that inositol 1,4,5-trisphosphate may be a second messenger mediating the effects of hormone on mobilization of intracellular Ca^{2+} . The relationship between mobilization of intracellular Ca^{2+} and Ca^{2+} gating is not known. It is clear that an early action of many hormones is to increase intracellular Ca^{2+} concentration through release of Ca^{2+} from intracellular storage sites. Hormones also stimulate the entry of extracellular Ca^{2+} , and this appears to be essential to augment and sustain the elevation of the concentration of cytosolic Ca^{2+} derived from intracellular stores. It cannot be assumed that the same parameters that affect intracellular Ca^{2+} mobilization also influence Ca^{2+} gating. Ca^{2+} gating refers to the trans-epithelial transport of Ca^{2+} across the salivary-gland membrane. This is a multi-step process involving a rate-limiting entry of extracellular Ca^{2+} across the basal membrane that is regulated by 5-HT. The Ca^{2+} that enters the cell is transported across the luminal membrane into the saliva, thus generating a transepithelial flux of Ca^{2+} (Berridge & Lipke, 1979). The major effect of 5-HT is to increase the permeability of the basal membrane to extracellular Ca^{2+} . Whether this effect is mediated through inositol 1,4,5-trisphos-

phate remains to be determined. It is possible that resynthesis of a critical amount of phosphoinositides is essential to maintain the receptor-regulated Ca^{2+} gate in a hormone-responsive state. Depletion of the hormone-sensitive pool of phosphoinositides during hormone stimulation may render the Ca^{2+} gate insensitive to receptor activation. Clearly, future studies need to be directed towards defining the relationship among mobilization of intracellular Ca^{2+} , Ca^{2+} gating and phosphoinositide breakdown.

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