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Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol

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The agonist-dependent hydrolysis of inositol phospholipids was investigated by studying the breakdown of prelabelled lipid or by measuring the accumulation of inositol phosphates. Stimulation of insect salivary glands with 5-hydroxytryptamine for 6 min provoked a rapid disappearance of [^{3}H]phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] and [^{3}H]phosphatidylinositol 4-phosphate (PtdIns4P) but had no effect on the level of [^{3}H]phosphatidylinositol (PtdIns). The breakdown of PtdIns(4,5)P_2 was associated with a very rapid release of inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], which reached a peak 5½ times that of the resting level after 5 s of stimulation. This high level was not maintained but declined to a lower level, perhaps reflecting the disappearance of PtdIns(4,5)P_2. 5-Hydroxytryptamine also induced a rapid and massive accumulation of inositol 1,4-bisphosphate [Ins(1,4)P_2]. The fact that these increases in Ins(1,4,5)P_3 and Ins(1,4)P_2 precede in time any increase in the level of inositol 1-phosphate or inositol provides a clear indication that the primary action of 5-hydroxytryptamine is to stimulate the hydrolysis of PtdIns(4,5)P_2 to yield diacylglycerol and Ins(1,4,5)P_3. The latter is then hydrolysed by a series of phosphomonoesterases to produce Ins(1,4)P_2, Ins1P and finally inositol. The very rapid agonist-dependent increases in Ins(1,4,5)P_3 and Ins(1,4)P_2 suggests that they could function as second messengers, perhaps to control the release of calcium from internal pools. The PtdIns(4,5)P_2 that is used by the receptor mechanism represents a small hormone-sensitive pool that must be constantly replenished by phosphorylation of PtdIns. Small changes in the size of this small energy-dependent pool of polyphosphoinositide will alter the effectiveness of the receptor mechanism and could account for phenomena such as desensitization and super-sensitivity.

An early response of cells to external signals is a rapid breakdown of a class of lipids known collectively as the phosphoinositides (Michell, 1975; Hawthorne & Pickard, 1979; Berridge, 1981; Putney, 1981). These phosphoinositides are closely related metabolically and there are several ways in which an agonist might reduce their level within the membrane. The major component of these lipids is PtdIns, which may be considered as the parent molecule from which the related polyphosphoinositides are formed by phosphorylation of its inositol head group. Phosphorylation of the hydroxy group on the 4-position of the inositol ring yields PtdIns4P, and the addition of another phosphate at the 5-position yields PtdIns(4,5)P_2. These phosphorylation steps can be reversed by phosphomonoesterases that convert these polyphosphoinositides back into PtdIns. In addition to the phosphorylation–dephosphorylation reactions, there are also phosphodiesterase enzymes that cleave off the inositol phosphate head groups.

When cells are incubated with [^{32}P]P_i or [^{3}H]-inositol, label is incorporated into the inositol phosphate head group of these phosphoinositides. Upon stimulation with agonists, there is a reduction in the level of label both in PtdIns and in the polyphosphoinositides (Abdel-Latif *et al.*, 1977; Hokin-Neaverson, 1977; Billah & Michell, 1979;

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)-P_2, phosphatidylinositol 4,5-bisphosphate; Ins1P, L-*myo*-inositol 1-phosphate; Ins(1,4)P_2, L-*myo*-inositol 1,4-bisphosphate; Ins(1,4,5)P_3, L-*myo*-inositol 1,4,5-trisphosphate.

Fain & Berridge, 1979; Akhtar & Abdel-Latif, 1980; Kirk *et al.*, 1981; Billah & Lapetina, 1982; Weiss *et al.*, 1982). There is a phosphodiesterase that acts on phosphatidylinositol to liberate a mixture of inositol 1:2-cyclic phosphate and Ins1*P* (Shukla, 1982). There is a separate phosphodiesterase acting on PtdIns4*P* and PtdIns(4,5)P_2 to liberate Ins(1,4)P_2 and Ins(1,4,5)P_3 respectively (Downes & Michell, 1981). Since these phosphoinositides are metabolically related to each other, the agonist-dependent reduction in the level of all three lipids could arise through the action of either one or both of these phosphodiesterases. In other words, agonists might induce the hydrolysis of all three lipids simultaneously or they may specifically hydrolyse one form, which then affects the levels of the other two through the phosphorylation–dephosphorylation system described above.

One way of trying to resolve this question of which of these phosphoinositides is the primary substrate used by the receptor mechanism is to carry out a kinetic analysis of the rate at which the water-soluble inositol phosphates are released when cells are stimulated. The salivary glands of the blowfly contain glycerophosphoinositol, Ins1*P*, Ins(1,4)P_2 and Ins(1,4,5)P_3. Upon stimulation with 5-hydroxytryptamine, there was a very large rise in the level of Ins(1,4)P_2, with smaller increases in Ins1*P* and Ins(1,4,5)P_3 (Berridge *et al.*, 1983). The time course of these events has been explored further and reveals that the first changes that occur upon stimulation are large increases in the levels of Ins(1,4,5)P_3 and Ins(1,4)P_2. This result shows that the polyphosphoinositides [particularly PtdIns-(4,5)P_2] may be the primary substrate hydrolysed upon activation of calcium-mobilizing receptors.

Materials and methods

Incubation of salivary glands

The abdominal portions of the salivary glands from adult female blowflies were isolated and incubated in a medium containing 20 μM-*myo*-[2-^{3}H]inositol (sp. radioactivity 9.3 Ci/mmol) for 2 h. In a typical experiment, 120 glands were incubated in 800 μl of medium containing the label. At the end of the 2 h incubation period, the label was removed and the glands were rinsed five times with fresh medium over a 30 min period. Groups of five glands were incubated at 30°C in 200 μl of control medium contained in small glass vials. The medium surrounding the glands was removed and replaced with a further 200 μl of either control medium or medium containing 5-hydroxytryptamine. This incubation was terminated by the addition of 1 ml of chloroform/methanol (1:2, v/v).

Extraction of lipid- and water-soluble metabolites

The chloroform/methanol extract of the labelled glands was transferred to a test tube. The glands were then homogenized in 200 μl of 0.5 M-HCl, which was added to the chloroform/methanol extract, together with a further 200 μl of water that was used to wash out the homogenizer. After addition of 200 μl of chloroform the tubes were shaken and then centrifuged.

After collecting the upper phase, the interface was washed twice with 200 μl of an upper phase prepared by mixing chloroform/methanol/0.1 M-sodium cyclohexane-1,2-diaminetetra-acetate (16:8:5, by vol.). The upper phase and washings were then dried and stored at −15°C before the separation of radioactive metabolites by anion-exchange chromatography. The lower phase containing the lipids was also dried before carrying out the deacylation procedure.

Lipid deacylation

Lipids were deacylated by using a transacylation procedure described by Clarke & Dawson (1981) whereby the fatty acids are transferred to methylamine with the release of the corresponding glycero-derivatives. The dried lipid extract was incubated for 30 min at 53°C with 0.75 ml of methylamine reagent containing monomethylamine/methanol/water/butanol (5:4:3:1, by vol.). At the end of the incubation, the methylamine reagent was removed by evaporation. The dry residue was taken up in 1 ml of water and fatty acids were removed by adding 1.2 ml of a mixture containing n-butanol/light petroleum (b.p. 40–60°C)/ethyl formate (20:4:1, by vol.). After shaking, the upper phase was removed and the lower phase was rewashed with 0.75 ml of the same solvent. The lower aqueous phase was applied to anion-exchange columns to separate the water-soluble deacylation products.

Anion-exchange chromatography

The water-soluble inositol phosphates were separated from each other by using small columns made from Pasteur pipettes containing 1 ml of Dowex-1 (X8; formate form; Sigma Chemical Co., London, S.W.6, U.K.) (Downes & Michell, 1981). The solutions used to elute off the different phosphates varied depending on which extract was being studied.

The radioactive phosphate esters extracted from the cell were eluted sequentially by using 5 mM-disodium tetraborate/60 mM-sodium formate (for glycerophosphoinositol), 0.1 M-formic acid/0.2 M-ammonium formate (for Ins1*P*), 0.1 M-formic acid/0.4 M-ammonium formate [for Ins(1,4)P_2] and 0.1 M-formic acid/1.0 M-ammonium formate [for Ins(1,4,5)P_3] as described by Berridge *et al.* (1983). Free inositol was not retained on the column and

thus appeared in the fractions collected at the beginning when the extract was being applied to the column. The column was washed through with approx. 10ml of distilled water to wash out all free inositol before eluting off the inositol phosphates.

The radioactive deacylation products were separated from each other by using 5mM-disodium tetraborate/0.18M-ammonium formate (for glycerophosphoinositol), 0.1M-formic acid/0.3M-ammonium formate (for glycerophosphoinositol 4-phosphate) and 0.1M-formic acid/0.75M-ammonium formate (for glycerophosphoinositol 4,5-bisphosphate) as described by Downes & Michell (1981).

Results

Effect of 5-hydroxytryptamine on the breakdown of inositol-containing phospholipids

When salivary glands were incubated with [^{3}H]inositol, label was incorporated into all three of the phosphoinositides. A quantitative estimate of how much label was present in each of these lipids was obtained by measuring the water-soluble products that are released when the fatty acid chains have been removed (Fig. 1). Most of the label (90%) was recovered as glycerophosphoinositol derived from phosphatidylinositol, and the remaining 10% was distributed between the two polyphosphoinositides. Approx. 7% of the label appeared as glycerophosphoinositol 4,5-bisphosphate derived from PtdIns(4,5)P_2, and the remaining 3% was glycerophosphoinositol 4-phosphate derived from PtdIns4P. The measurement of these glycerophosphoinositol derivatives provided an accurate and reliable method for studying the effect of 5-hydroxytryptamine on these inositol-containing phospholipids.

When salivary glands that had been prelabelled with [^{3}H]inositol were stimulated with 10μM-5-hydroxytryptamine, there was a significant decline in the labelled PtdIns(4,5)P_2 and PtdIns4P but no significant change in PtdIns (Fig. 2). It is apparent from the error bars that the amount of label incorporated into these phospholipids was extremely variable, which means that it was very difficult to detect changes during early time periods. For this reason, the release of inositol phosphates as a measure of phospholipid breakdown was investigated.

Time course of the effect of 5-hydroxytryptamine on the accumulation of inositol and inositol phosphates

Apart from free inositol, the blowfly salivary gland contains glycerophosphoinositol, Ins1P, Ins(1,4)P_2 and Ins(1,4,5)P_3 (Berridge *et al.*, 1983). These metabolites can be separated from each other by using anion-exchange chromatography. When salivary glands that had been prelabelled with [^{3}H]inositol were stimulated with 5-hydroxytryptamine there were large changes in the radioactivity associated with some of these metabolites.

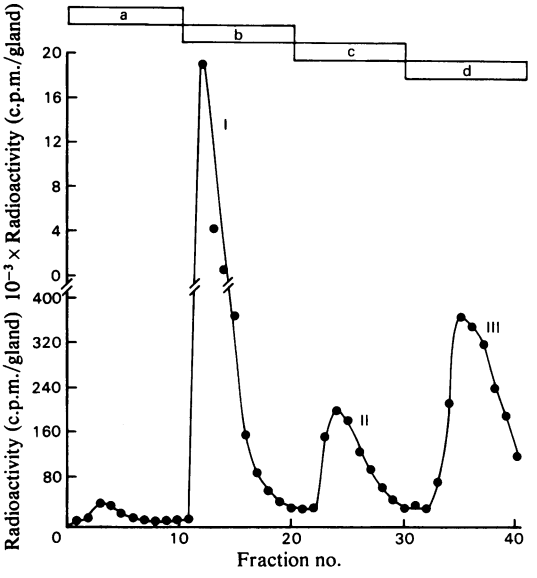

Fig. 1. *The separation of water-soluble metabolites obtained by the deacylation of phosphoinositides extracted from the blowfly salivary gland*

A group of 15 glands was incubated in 50μl of 20μM-[^{3}H]inositol for 2h. After washing out the label the phosphoinositides were extracted from the glands and deacylated as described in the Materials and methods section. The water-soluble deacylation products were applied to anion-exchange columns and eluted sequentially with (a) distilled water (removes a small amount of free inositol, (b) 5mM-disodium tetraborate/0.18M-ammonium formate (for glycerophosphoinositol; peak I), (c) 0.1M-formic acid/0.3M-ammonium formate (for glycerophosphoinositol 4-phosphate; peak II) and (d) 0.1M-formic acid/0.75M-ammonium formate (for glycerophosphoinositol 4,5-bisphosphate; peak III).

Inositol

The level of free [^{3}H]inositol in unstimulated glands changed very little over a period of 15min (Fig. 3*a*). However, upon stimulation with 5-hydroxytryptamine there was a linear increase in the accumulation of [^{3}H]inositol. Most of this [^{3}H]inositol was released to the bathing medium and this efflux of labelled inositol was used in previous studies as an indirect measure of the rate of breakdown of PtdIns (Fain & Berridge, 1979). However, the first products of phosphoinositide breakdown are the inositol phosphates and not

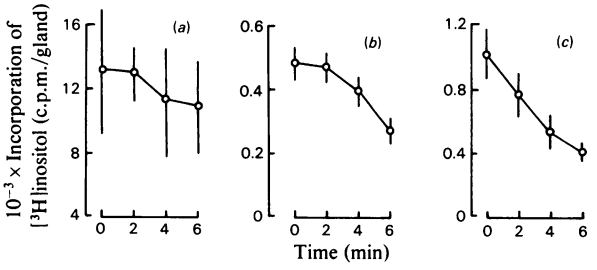

Fig. 2. *Effect of 5-hydroxytryptamine on the level of (a) PtdIns, (b) PtdIns4P and (c) PtdIns(4,5)P_2 in the blowfly salivary gland*

A group of 120 glands was incubated in 1 ml of medium containing 20 μM-[^{3}H]inositol for 2 h. After washing out the label, groups of 5 glands were stimulated with 200 μl of 5-hydroxytryptamine (10 μM) at 30°C. The incubations were stopped after various times by addition of 1 ml of chloroform/methanol (1:2, v/v). After extraction and deacylation of the phosphoinositides, the water-soluble products were separated as shown in Figure 1. The amount of label present in each phospholipid is expressed as c.p.m./gland. Each point represents the mean ± S.E.M. ($n = 6$).

Table 1. *Effect of 5-hydroxytryptamine on the accumulation of [^{3}H]Ins1P in the blowfly salivary gland*

The incubation and extraction techniques were identical with those used for the experiment described in the legend to Fig. 3. 5-Hydroxytryptamine (10 μM) was added at time zero and the amount of label present as [^{3}H]Ins1*P* is expressed as c.p.m./gland (mean ± S.E.M.; $n = 6$). Experiments carried out on the same batch of flies are identified by the superscript letters. *$P < 0.01$.

Time	Radioactivity (c.p.m./gland) Control	5-Hydroxytryptamine	Stimulation by 5-hydroxytryptamine (%)
10 s[a]	294 ± 13[a]	273 ± 31	93
20 s[a]	304 ± 9[a]	334 ± 44	110
45 s[b]	480 ± 71[b]	564 ± 124	118
60 s	130 ± 21	250 ± 10*	192
1.5 min	196 ± 25	462 ± 9.0*	236
2 min[c]	95 ± 13[c]	329 ± 36*	346
4 min[b]	371 ± 18[b]	646 ± 88*	174
8 min[c]	98 ± 12[c]	356 ± 60*	363
15 min[c]	93 ± 11[c]	300 ± 68*	323

inositol. It is noteworthy, therefore, that there is a 10–20 s lag period before the [^{3}H]inositol begins to accumulate (Fig. 3*a*).

Glycerophosphoinositol

This deacylation product of PtdIns was always present in the tissue extracts, but it did not change when glands were stimulated with 5-hydroxytrypt-

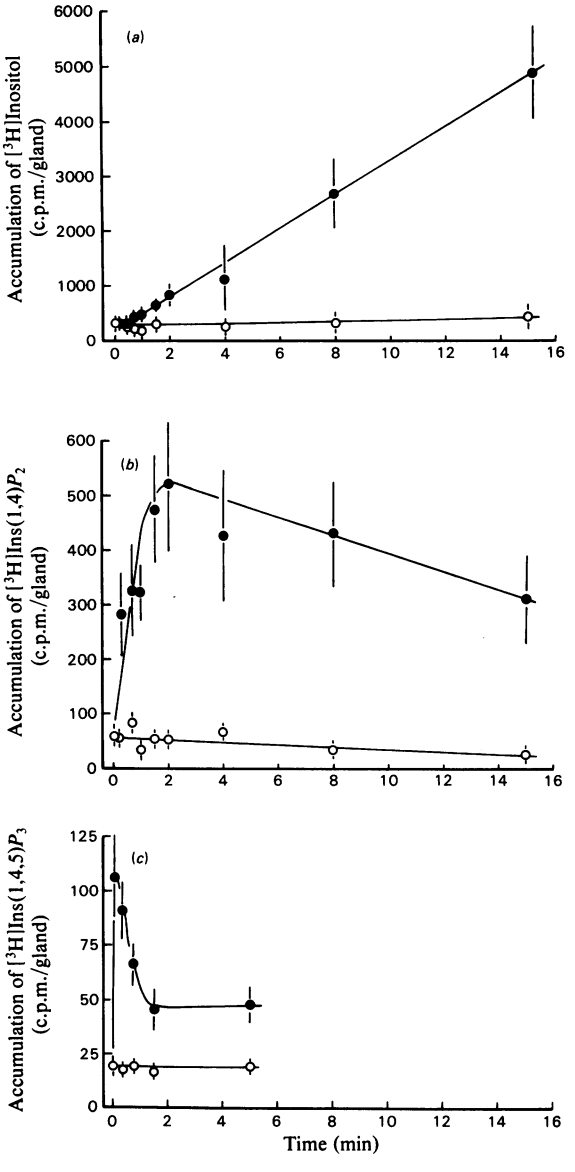

Fig. 3. *Effect of 5-hydroxytryptamine on the accumulation of [^{3}H]inositol (a) and on the intracellular level of [^{3}H]Ins(1,4)P_2 (b) and [^{3}H]Ins(1,4,5)P_3 (c) in the blowfly salivary gland*

Groups of five salivary glands that had been prelabelled for 2 h with [^{3}H]inositol were carefully washed before being incubated for various times in 200 μl of medium either in the absence (○) or in the presence of 10 μM-5-hydroxytryptamine (●) added at time zero. The incubations were stopped by the addition of 1 ml of chloroform/methanol (1:2, v/v) and water-soluble metabolites were extracted and separated from each other by anion-exchange chromatography (see the Materials and methods section). The amount of label present in the different metabolites is expressed as c.p.m./gland. Each point is the mean ± S.E.M. ($n = 6$).

amine (results not shown). A previous study revealed the presence of an enzyme that can deacylate PtdIns and could be responsible for the formation of this inositol phosphate (Irvine *et al.*, 1982). The fact that glycerophosphoinositol does not change upon stimulation suggests that the deacylation enzyme may not play a direct role in the action of 5-hydroxytryptamine.

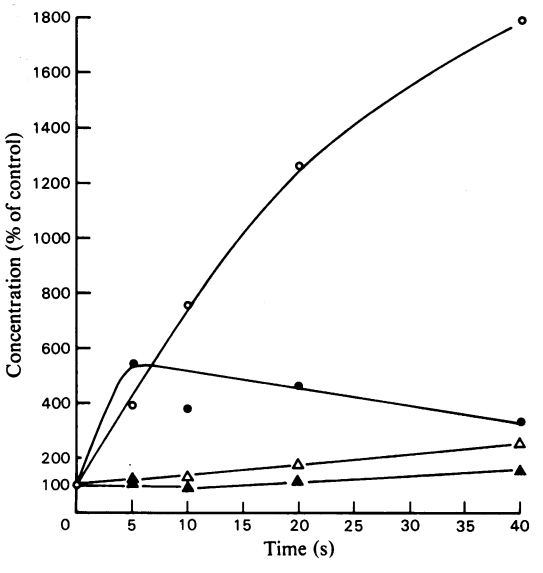

Fig. 4. *Time course of the changes in [^{3}H]inositol and [^{3}H]inositol phosphates induced by 5-hydroxytryptamine*
The change in the concentration of the different metabolites has been expressed as a percentage of the control value obtained just before the addition of 5-hydroxytryptamine (10 μM). Most of the values were obtained from the data contained in Table 2 and Fig. 3. △, [^{3}H]inositol; ▲, [^{3}H]Ins1*P*; ○, [^{3}H]Ins(1,4)P_2; ●, [^{3}H]Ins(1,4,5)P_3.

Ins1P

The amount of [^{3}H]Ins1*P* in prelabelled salivary glands varied considerably between experimental groups (Table 1). 5-Hydroxytryptamine induced a significant increase in the level of [^{3}H]Ins1*P* 1 min after stimulation. At the earlier time periods, there was very little change in the level of this monophosphate as shown in Fig. 4.

Ins(1,4)P_2

5-Hydroxytryptamine induced a very large and rapid increase in the accumulation of [^{3}H]Ins(1,4)P_2 (Fig. 3*b*). At the peak of the response, the level of [^{3}H]Ins(1,4)P_2 was 10 times the control value. When the change in this bisphosphate was studied over a short time period, there appeared to be no lag period (Fig. 4). After 5 s of stimulation, the concentration of [^{3}H]Ins(1,4)P_2 had increased 4-fold over the resting level, whereas there was little or no change in the levels in either inositol or Ins1*P* (Fig. 4).

Ins(1,4,5)P_3

The effect of 5-hydroxytryptamine on [^{3}H]Ins-(1,4,5)P_3 was distinctly biphasic (Fig. 3*c*). The peak response, which represented a 5.5-fold increase in the level of this trisphosphate, occurred at 5 s, after which it declined to a plateau value just over twice that of the resting level. The fact that [^{3}H]Ins(1,4,5)P_3 does not go on accumulating suggests that it is rapidly hydrolysed to [^{3}H]Ins(1,4)P_2 by a specific phosphomonoesterase. The phosphomonoesterase that converts Ins(1,4)P_2 into Ins1*P* is apparently much less active because there was a very large accumulation of [^{3}H]Ins-(1,4)P_2 that precedes any increase in the level of Ins1*P* or inositol (Fig. 4).

Table 2. *Effect of 5-hydroxytryptamine on the breakdown of phosphoinositides and the increase in inositol phosphates*
Groups of five salivary glands that had been prelabelled for 2 h in [^{3}H]inositol were carefully washed before being incubated for 2 min in 200 μl of medium either in the absence or presence of 10 μM-5-hydroxytryptamine. The incubations were stopped by the addition of 1 ml of chloroform/methanol (1 : 2, v/v). The lipids and the water-soluble metabolites were extracted and measured as described in the Materials and methods section. The amount of radioactivity present in each compound is expressed as c.p.m./gland (means ± S.E.M.). *$P < 0.05$; **$P < 0.01$.

	Control (c.p.m./gland)	5-Hydroxytryptamine (c.p.m./gland)	Decrease in lipid (c.p.m.)	Increase in water-soluble metabolites (c.p.m.)
Lipid				
PtdIns	21307 ± 2960	21218 ± 2105	89	—
PtdIns4*P*	834 ± 84	796 ± 77	38	—
PtdIns(4,5)P_2	1381 ± 256	1016 ± 111*	365	—
Water-soluble metabolite				
Ins(1,4,5)P_3	69 ± 10	110 ± 10**	—	41
Ins(1,4)P_2	28 ± 7	522 ± 63**	—	494
Ins1*P*	93 ± 53	329 ± 36**	—	236
Inositol	266 ± 28	840 ± 81**	—	574

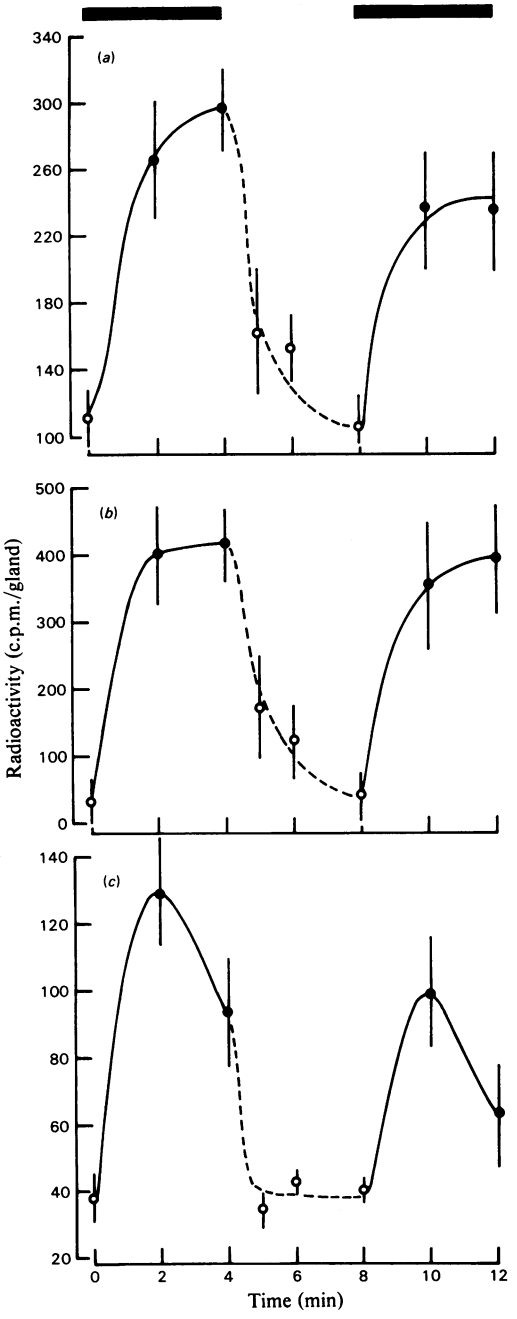

Fig. 5. *Effect of removing 5-hydroxytryptamine on the rate of recovery of the inositol phosphates*

The incubation and extraction techniques were identical with those used for the experiments described in Fig. 3. Groups of five salivary glands were stimulated with 10 μM-5-hydroxytryptamine for the periods shown by the horizontal bars. From 4 to 8 min, the 5-hydroxytryptamine was removed and replaced with control medium containing 100 μM-gramine (a 5-hydroxytryptamine receptor antagonist). The amount of label present as [^{3}H]Ins1*P* (*a*), [^{3}H]Ins(1,4)P_2, (*b*) and [^{3}H]Ins(1,4,5)P_3 (*c*) is expressed as c.p.m./gland. Each point represents the mean $\pm$ S.E.M. ($n = 6$).

Comparison of effects of 5-hydroxytryptamine on the labelling of lipid- and water-soluble compounds

To establish the relationship between the decrease in lipid labelling with the increase in water-soluble metabolites, both aspects were measured on the same group of glands after a 2 min stimulation with 10 μM-5-hydroxytryptamine (Table 2). The total label lost from PtdIns4*P* and PtdIns(4,5)P_2 was 403 c.p.m., which was somewhat smaller than the increase in total label in Ins(1,4,5)P_3 and Ins(1,4)P_2 (535 c.p.m.). The latter is probably an underestimate of the amount of label that had been released from the polyphosphoinositides because some of the Ins(1,4)P_2 will have been hydrolysed to Ins1*P* and free inositol during the 2 min period of stimulation. If the increase in label in Ins1*P* and free inositol is included, then the total increase in water-soluble label is 1345 c.p.m., which greatly exceeds the loss of label from the polyphosphoinositides. This discrepancy may be explained by assuming either that label is also released from PtdIns or that the polyphosphoinositides are turning over quickly and are constantly being replaced by phosphorylation of PtdIns.

Reversibility of these 5-hydroxytryptamine-dependent changes in inositol phosphates

The inositol phosphates that are released upon stimulation must be rapidly dephosphorylated to produce the large amount of inositol that accumulates during prolonged stimulation (Fig. 3*a*). To demonstrate that these metabolites are in a dynamic equilibrium, their rate of recovery upon removal of 5-hydroxytryptamine was investigated (Fig. 5). Upon application of 5-hydroxytryptamine there was the usual increase in the levels of [^{3}H]Ins1*P*, [^{3}H]Ins(1,4)P_2 and [^{3}H]Ins(1,4,5)P_3. When the stimulus was withdrawn, there was a rapid return to the unstimulated level. When glands were restimulated with 5-hydroxytryptamine, the levels of all these inositol phosphates increased once again (Fig. 5).

Discussion

The main object of the present study was to identify which of the three inositol phospholipids is used as a primary substrate at a calcium-mobilizing receptor. Previous studies had shown that when the blowfly salivary gland was incubated with [^{3}H]inositol, label was incorporated not only into PtdIns but

also into the polyphosphoinositides (Fain & Berridge, 1979). Upon stimulation with 5-hydroxytryptamine for 30 min, more than 75% of this label was released from both forms. When this breakdown of prelabelled lipids was studied over shorter time intervals as shown in Fig. 2, the earliest and most significant change was seen for the polyphosphoinositides, whereas there was no significant change in PtdIns. The ability of agonists to induce a rapid reduction in the level of the polyphosphoinositides [particularly PtdIns(4,5)P_2] has already been described in rabbit iris muscle (Abdel-Latif *et al.*, 1977) in rat hepatocytes (Kirk *et al.*, 1981; Michell *et al.*, 1981), in parotid gland (Weiss *et al.*, 1982) and in blood platelets (Billah & Lapetina, 1982). Although such observations clearly suggest that the primary action of agonists is to stimulate a decrease in the level of the polyphosphoinositides, they provide no information concerning which pathway is used to degrade these inositol lipids. The existence of different pathways for the metabolism of these inositol phospholipids stems from the fact that they are readily interconvertible through phosphomonoesterases that dephosphorylate the polyphosphoinositides and kinases that reverse this process by phosphorylating PtdIns to the two polyphosphoinositides. The existence of this interconnected system suggests two main mechanisms whereby agonists might induce a large decrease in the level of the polyphosphoinositides. One possible mechanism would depend upon the action of the phosphomonoesterases that sequentially remove phosphate from the 5- and 4-position to give PtdIns (Fig. 6*a*). In this mechanism, the primary substrate used by the receptor mechanism would be PtdIns, which would be degraded by a phosphodiesterase that yields diacylglycerol and a mixture of Ins1*P* and inositol 1:2-cyclic phosphate when studied *in vitro* (Shukla, 1982). The alternative pathway, which could also lead to a decrease in the level of the polyphosphoinositides, involves a phosphodiesterase that acts directly on PtdIns(4,5)P_2 to form diacylglycerol and Ins(1,4,5)P_3 (Fig. 6*b*). The latter would then be degraded sequentially through a series of phosphatases to inositol(1,4)P_2, inositol1*P* and inositol. The situation regarding PtdIns4*P* is a little more complicated in that it could either be hydrolysed directly by a similar phosphodiesterase to give diacylglycerol and Ins(1,4)P_2 or it could first be converted into PtdIns(4,5)P_2 before hydrolysis as depicted in Fig. 6(*b*). The former seems unlikely because there was no breakdown of PtdIns4*P* over the first 2 min of stimulation by 5-hydroxytryptamine (Fig. 2). For the sake of simplicity, therefore, it will be assumed that Ins(1,4)P_2 is derived solely from Ins(1,4,5)P_3, bearing in mind that the inositol bisphosphate may also be derived directly by the hydrolysis of PtdIns4*P*. The main point is that the agonist acts primarily to hydrolyse the polyphosphoinositides rather than PtdIns. In this mechanism, PtdIns functions indirectly as a reservoir of precursor molecules that are phosphorylated to form the polyphosphoinositides used by the receptor transducing mechanism. The main difference between these two pathways, therefore, depends on whether PtdIns or PtdIns(4,5)P_2 is used as the primary substrate for the receptor mechanism.

One way of determining which inositol phospholipid is used by the receptor mechanism is to examine the water-soluble products resulting from the hydrolysis of either PtdIns or the polyphosphoinositides. In the former case, the hydrolysis of PtdIns will yield Ins1*P* and inositol 1:2-cyclic phosphate, whereas the hydrolysis of the polyphoinositides will produce Ins(1,4,5)P_3 and Ins(1,4)P_2. Both Ins(1,4)P_2 and Ins(1,4,5)P_3 have been identified in this insect salivary gland and in mammalian brain and parotid gland (Berridge *et al.*, 1983). Other studies have also revealed the presence of the inositol bis- and tris-phosphate in guinea-pig synaptosomes (Griffin & Hawthorne, 1978) and also in rabbit iris smooth muscle (Akhtar & Abdel-Latif, 1980). Using homogenates of guinea-pig brain, Durell *et al.* (1968) demonstrated that acetylcholine stimulated an increase in the formation of Ins1*P* and Ins(1,4)P_2. The ionophore A23187 caused a similar response in synaptosomes, with the increase in Ins(1,4)P_2 being the dominant effect (Griffin & Hawthorne, 1978). In rabbit iris smooth muscle, however, the major action of acetylcholine was to increase Ins(1,4,5)P_3 and Ins1*P* (Akhtar & Abdel-Latif, 1980). In a previous study on the insect salivary gland, 5-hydroxytryptamine was found to markedly increase the level of Ins(1,4)P_2, with a smaller change in Ins(1,4,5)P_3 (Berridge *et al.*, 1983). However, there also was a significant increase in the level of Ins1*P*, which could have arisen either from the hydrolysis of PtdIns or it could have resulted from the dephosphorylation of Ins(1,4,5)P_3 and Ins(1,4)P_2 produced from the hydrolysis of PtdIns(4,5)P_2 (Fig. 6*b*). To decide between these alternatives, it was necessary to carry out a kinetic analysis of the early agonist-dependent changes in the levels of these inositol phosphates. Over the first 5 s of stimulation with 5-hydroxytryptamine, there was a large increase in the levels of Ins(1,4,5)P_3 and Ins(1,4)P_2, whereas there was no change in the level of either Ins1*P* or free inositol. The last two products were found to increase much later. This very marked difference in the time course of events seems to support the scheme outlined in Fig. 6(*b*) whereby 5-hydroxytryptamine acts by stimulating the hydrolysis of PtdIns(4,5)P_2 to yield diacylglycerol and Ins(1,4,5)P_3. The latter then enters an inositol phosphate cycle whereby it undergoes a stepwise

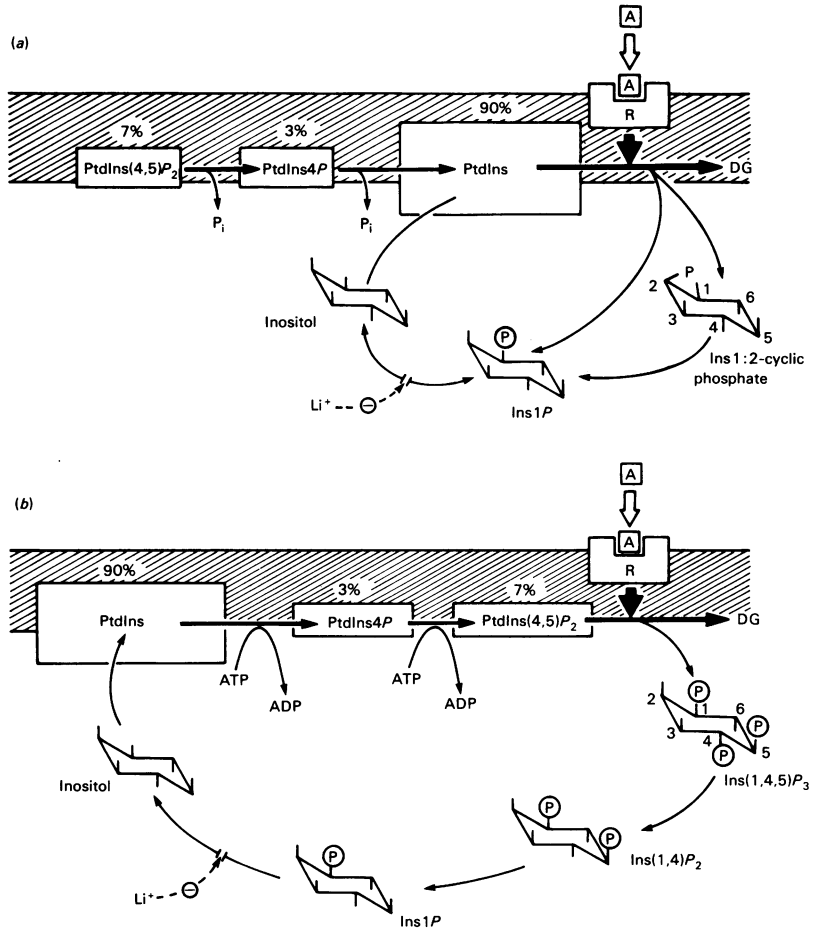

Fig. 6. *Two alternative mechanisms for the agonist (A)-dependent hydrolysis of membrane phosphoinositides by Ca^{2+}-mobilizing receptors*

(*a*) The primary substrate for the receptor (R) mechanism is PtdIns that is hydrolysed by a phosphodiesterase to yield diacylglycerol (DG) and a mixture of inositol 1:2-cyclic phosphate and Ins1*P*. The polyphosphoinositides [PtdIns(4,5)P_2 and PtdIns4*P*] could also be used by the receptor mechanism once they have been converted into PtdIns by dephosphorylation. (*b*) In this case, the primary substrate for the receptor mechanism is PtdIns(4,5)P_2 that is hydrolysed to DG and Ins(1,4,5)P_3 which then enters an inositol phosphate cycle during which the three phosphates are removed sequentially to liberate the inositol required to resynthesize PtdIns. The final step of this cycle, the dephosphorylation of Ins1*P* to inositol, is inhibited by lithium. The PtdIns serves as a reservoir to supply precursors that can be phosphorylated to maintain the small hormone-sensitive PtdIns(4,5)P_2 pool. The observation that 5-hydroxytryptamine stimulated a rapid increase in the level of Ins(1,4,5)P_3 and Ins(1,4)P_2 would favour model (*b*) over model (*a*).

dephosphorylation first to Ins(1,4)P_2, then to Ins1*P* and finally to inositol, which is used to resynthesize PtdIns. The flux of metabolites through this cycle is totally agonist-dependent because when 5-hydroxytryptamine is withdrawn, the stimulated levels of all three inositol phosphates returned rapidly to their resting levels (Fig. 5). Upon restimulation, they all increased again. The fact that 5-hydroxytryptamine acts initially to increase the levels of the first two components of this cycle clearly suggests that the hydrolysis of PtdIns(4,5)P_2 is an integral part of the transducing mechanism of calcium-mobilizing receptors.

The very large and rapid increases in the levels of Ins(1,4,5)P_3 and Ins(1,4)P_2 that have been observed in response to 5-hydroxytryptamine raise the interesting possibility that one or other of these two inositol phosphates could function as a second

messenger. They have all the attributes of a classical second messenger such as cyclic AMP in that they are generated rapidly upon arrival of the agonist and are destroyed equally as fast when the agonist is removed. The problem is to ascribe a role for these putative second messengers. One possibility is that they may function to mobilize calcium from internal reservoirs. In a number of tissues, agonists acting on surface receptors can bring about a release of calcium from some internal reservoir as occurs during stimulation of the pancreas with acetylcholine (Schulz & Stolze, 1980) or when the liver is stimulated by α-adrenergic agents (Exton, 1981). A similar phenomenon has been described in the insect salivary gland, where 5-hydroxytryptamine can trigger repeated calcium-dependent membrane depolarizations in the complete absence of external calcium (Berridge & Prince, 1975). The action of all these calcium-mobilizing receptors is associated with the hydrolysis of inositol phospholipids that generate inositol phosphates that might function as second messengers to link surface receptors with internal organelles.

There are a number of very interesting consequences of using PtdIns(4,5)P_2 as a substrate for a receptor-transducing mechanism. In the first place, the polyphosphoinositides represent a very small pool that must be continuously replenished from PtdIns using energy-dependent processes. As this small hormone-sensitive pool is used up by the receptor mechanism, it must be replaced by two phosphorylation reactions (Fig. 6*b*), which means that the operation of these calcium-mobilizing receptors should be very sensitive to the intracellular level of ATP. In pancreas (Hokin, 1974) and blood platelets (Holmsen *et al.*, 1982) the agonist-dependent breakdown of PtdIns was blocked by metabolic inhibitors, which led to the suggestion that there was an energy-requiring step for the activation of phospholipase C. However, on the basis of the experiments described in the present paper, the energy-requiring step can be identified as the phosphorylation reaction that converts PtdIns into the PtdIns(4,5)P_2 used by the receptor mechanism. Evidence for such an ATP-dependent requirement for the operation of calcium-mobilizing receptors has been obtained from studies on lymphocytes (Pozzan *et al.*, 1982). Mitochondrial poisons that lowered the level of ATP completely blocked the antigen-induced rise in intracellular calcium concentration. That the extrusion of calcium from the cell against its electrochemical gradient is an energy-dependent process is well-established. It now seems that the onset of the calcium signal induced by these agonist-dependent receptors may also require energy. The fact that both the entry as well as the extrusion of calcium are linked to ATP makes physiological sense because it will prevent the cell from overloading itself with calcium when low ATP levels inhibit calcium extrusion. In other words, the entry of calcium through agonist-dependent channels will be automatically coupled to the ability of the cell to extrude calcium by having both processes linked together through ATP.

The existence of such a small energy-dependent pool of PtdIns(4,5)P_2 suggests a possible mechanism for adjusting receptor sensitivity by varying the availability of this substrate. Any reduction in the level of PtdIns(4,5)P_2 will reduce the effectiveness of the receptor mechanism and could account for the phenomenon of desensitization, which is a characteristic feature of calcium-mobilizing receptors (Berridge, 1981).

In summary, an analysis of changes in the levels of inositol phosphates in the insect salivary gland in response to 5-hydroxytryptamine clearly shows that the primary action of this neurohormone is to stimulate the hydrolysis of the polyphosphoinositides. On the basis of the evidence obtained so far, it is not possible to determine whether PtdIns4*P* is also used as a substrate by the receptor mechanism or whether it is used indirectly by being converted into PtdIns(4,5)P_2. In this model, PtdIns has no direct role to play in the receptor mechanism other than to function as a reservoir to replenish the small hormone-sensitive pool of polyphosphoinositides. Since the maintenance of this pool is energy-dependent, this implies that the function of calcium-mobilizing receptors will require a continuous supply of ATP. It also means that the effectiveness of these receptors could be adjusted through subtle modification in the phosphorylation–dephosphorylation processes that determine the size of this hormone-sensitive pool. An increase in the level of the polyphosphoinositides might account for supersensitivity, whereas a decrease would result in desensitization.

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References

Abdel-Latif, A. A., Akhtar, R. A. & Hawthorne, J. N. (1977) *Biochem. J.* **162**, 61–73

Abdel-Latif, A. A., Green, K. & Smith, J. P. (1979) *J. Neurochem.* **32**, 225–228

Akhtar, R. A. & Abdel-Latif, A. A. (1980) *Biochem. J.* **192**, 783–791

Berridge, M. J. (1981) *Mol. Cell. Endocrinol.* **24**, 115–140

Berridge, M. J. & Prince, W. T. (1975) *J. Cyclic Nucleotide Res.* **1**, 169–176

Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482

Billah, M. M. & Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 12705–12708
Billah, M. M. & Michell, R. H. (1979) *Biochem. J.* **182**, 661–668
Clarke, N. G. & Dawson, R. M. C. (1981) *Biochem. J.* **195**, 301–306
Downes, C. P. & Michell, R. H. (1981) *Biochem. J.* **198**, 133–140
Durell, J., Sodd, M. A. & Friedel, R. O. (1968) *Life Sci.* **7**, 363–368
Exton, J. H. (1981) *Mol. Cell. Endocrinol.* **23**, 233–264
Fain, J. N. & Berridge, M. J. (1979) *Biochem. J.* **178**, 45–58
Griffin, H. D. & Hawthorne, J. N. (1978) *Biochem. J.* **176**, 541–552
Hawthorne, J. N. & Pickard, M. R. (1978) *J. Neurochem.* **32**, 5–14
Hokin, M. R. (1974) in *Secretory Mechanisms of Exocrine Glands* (Thorn, N. A. & Petersen, O. H., eds.), pp. 101–112, Munksgaard, Copenhagen
Hokin-Neaverson, M. (1977) *Adv. Exp. Biol. Med.* **3**, 429–446
Holmsen, H., Kaplan, K. L. & Dangelmaier, C. A. (1982) *Biochem. J.* **208**, 9–18
Irvine, R. F., Berridge, M. J., Letcher, A. J. & Dawson, R. M. C. (1982) *Biochem. J.* **204**, 361–364
Kirk, C. J., Creba, J. A., Downes, C. P. & Michell, R. H. (1981) *Biochem. Soc. Trans.* **9**, 377–379
Pozzan, T., Arslan, P., Tsien, R. Y. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 335–340
Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
Michell, R. H., Kirk, C. J., Jones, L. M., Downes, C. P. & Creba, J. A. (1981) *Philos. Trans. R. Soc. London Ser. B.* **296**, 123–138
Putney, J. W. (1981) *Life Sci.* **29**, 1183–1194
Schulz, I. & Stolze, H. H. (1980) *Annu. Rev. Physiol.* **42**, 127–156
Shukla, S. C. (1982) *Life Sci.* **30**, 1323–1335
Weiss, S. J., McKinney, J. S. & Putney, J. W. (1982) *Biochem. J.* **206**, 555–560