Phosphatidylinositol-hydrolysing enzymes in blowfly salivary glands

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The salivary glands of adult blowflies (*Calliphora erythrocephala*) contain enzymes that hydrolyse phosphatidylinositol, predominantly by a Ca^{2+} -independent deacylation, though a Ca^{2+} -dependent phosphodiesterase (phospholipase C) activity could be detected. The deacylating enzymes could also hydrolyse phosphatidylcholine and phosphatidylethanolamine, and were secreted in the saliva. Homogenization of salivary glands prelabelled with [^3H]inositol resulted in a rapid deacylation of the endogenous ^3H -labelled phosphatidylinositol; this hydrolysis was unaffected by addition of 5-hydroxytryptamine to the homogenate.

The salivary gland of the adult blowfly (*Calliphora erythrocephala*) exhibits a specific stimulation of phosphatidylinositol turnover involving an enhanced catabolism when exposed to 5-hydroxytryptamine (Fain & Berridge, 1979a,b; Berridge & Fain, 1979). As a further step in our attempts to understand the regulation of this turnover, it is necessary to study the enzymes that hydrolyse phosphatidylinositol in these glands. In particular, we hoped to investigate the (presumed) blowfly phosphatidylinositol phosphodiesterase to see how it compares with the equivalent mammalian enzyme, particularly with respect to possible inhibitory and stimulatory mechanisms (Irvine *et al.*, 1979; Dawson *et al.*, 1980; Hirasawa *et al.*, 1981). An additional impetus to our investigation was a report by Litosch & Fain (1981) that 5-hydroxytryptamine was able to stimulate the hydrolysis of phosphatidylinositol by a Ca^{2+} -independent phospholipase C in blowfly gland homogenates.

Materials and methods

Incubation conditions

Salivary glands were isolated from adult female blowflies (*Calliphora erythrocephala*) as described previously (Fain & Berridge, 1979a). For the collection of saliva, six glands were set up in drops of buffer kept under liquid paraffin (Berridge & Patel, 1968). The glands were stimulated with $1\mu\text{M}$ -5-hydroxytryptamine and the resulting saliva was collected and analysed for enzyme activity.

Enzyme studies with exogenous substrate

A Potter–Elvehjem-type glass homogenizer was used to homogenize 30–60 glands in 1 ml of 0.32 M-sucrose. This homogenate was centrifuged at 100 000 g for 60 min and the resulting supernatant was used as an enzyme source.

The ^{32}P -labelled phospholipids used as substrates were prepared from baker's yeast (Irvine *et al.*, 1978). The method for obtaining [2- ^{14}C]oleoyl-glycerophosphoinositol was as described by Irvine & Dawson (1979). Incubations were performed at pH 7.0 with 3 mM- Ca^{2+} or 6 mM-EGTA unless stated otherwise.

Enzyme studies with endogenous substrate

Salivary glands were prelabelled by incubation in [^3H]inositol before homogenization as previously described (Fain & Berridge, 1979a); approx. 30 glands were incubated in 600 μl of medium containing 12.6×10^6 c.p.m. of *myo*-[2- ^3H]inositol (sp. radioactivity 5 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) for 90 min. The glands were homogenized at 0°C in a glass homogenizer in 200 μl of a medium containing 150 mM- K^+ , 2 mM-Tris/HCl and 5 mM-EGTA at pH 7.0. The contents were combined with a further 500 μl of medium used to wash out the homogenizer. This combined extract was kept cold and divided into 50 μl portions. These were then incubated at 30°C for various times either in the absence or in the presence of 20 μM -5-hydroxytryptamine. The reaction was stopped by adding 500 μl of chloroform/methanol (1:2, v/v).

In some experiments, the breakdown of endogenous substrate was studied by measuring the efflux of inositol or ethanolamine from prelabelled cells. Four glands were incubated in 50 μ l of medium (Fain & Berridge, 1979a) containing either *myo*-[2- 3 H]-inositol (1×10^6 c.p.m.) or [2- 14 C]ethanolamine (1.8×10^6 c.p.m.). At the end of 1 h, the glands were rinsed five times with fresh medium before beginning the efflux experiment. At 3 min intervals the medium surrounding the glands was removed and counted for radioactivity, and fresh medium was added to the glands to measure efflux over the next interval (for further details see Fain & Berridge, 1979a).

Analytical techniques

The water-soluble products of phosphatidylinositol hydrolysis were separated ionophoretically (Dawson & Clarke, 1972), with non-radioactive phosphoinositol, cyclic-1:2-phosphoinositol and glycerophosphoinositol as markers.

Lysophospholipids (and residual phospholipid substrates) were extracted as described by Bjerve *et al.* (1975), and separated by t.l.c. (Irvine *et al.*, 1980). Lysophosphatidylinositol was included in all samples as a carrier and check on recovery.

Results and discussion

Products of hydrolysis of phosphatidylinositol

Incubation of the supernatant from a blowfly salivary-gland homogenate with [32 P]phosphatidylinositol established that there was a very active deacylating activity capable of releasing glycerophosphoinositol. The latter compound was unequivocally identified by autoradiography of ionophoretograms and scintillation counting of marker spots as the major (>95%) water-soluble 32 P-labelled product of the hydrolysis at pH 6.8, in either the presence or absence of 3 mM- Ca^{2+} . Lysophosphatidylinositol was also identified, suggesting that the deacylation occurred stepwise with separate enzymes, as described below.

In addition to this major deacylating activity, in the presence of 3 mM- Ca^{2+} there was unequivocal evidence (by autoradiography and scintillation counting) for a small (approx. 5% of the deacylating activity) release of labelled phosphoinositol and cyclic-1:2-phosphoinositol from [32 P]-phosphatidylinositol, and of labelled diacylglycerol when [2- 14 C]oleoylglycerophosphoinositol was used as a substrate. None of these three products was released if 6 mM-EGTA was substituted for 3 mM- Ca^{2+} in the incubation. These results confirm the presence in this gland of a Ca^{2+} -dependent phosphatidylinositol phosphodiesterase (EC 3.1.4.10) (Fain & Berridge, 1979a), though the specificity of this phosphodiesterase with regard to other phospholipids was not examined; the absence of phos-

phodiesterase products if EGTA is present indicates that we found no evidence for a Ca^{2+} -independent phosphatidylinositol phosphodiesterase (Litosch & Fain, 1981).

pH-dependence of the deacylating activity

The ratio of lysophosphatidylinositol to glycerophosphoinositol varied with pH (Fig. 1), which

Fig. 1. Effect of pH on phosphatidylinositol-deacylating enzymes in blowfly salivary glands

A fly salivary-gland homogenate supernatant (see the Materials and methods section) was incubated with 65 nmol of [32 P]phosphatidylinositol for 30 min at 37°C in the presence of 10 mM-EGTA. The pH was varied by using 0.05 M- (final concentration) Tris/maleate/NaOH buffers. After separation of two phases by adding butanol (see the Materials and methods section), and washing of the lower phase with butanol, a sample of the lower phase was counted for radioactivity. Separate experiments showed that over 99% of this radioactivity was in glycerophosphoinositol. The upper phase was reduced in volume and the lysophosphatidylinositol separated by t.l.c. (see the Materials and methods section). Confirmatory results were obtained in another experiment. Two lines are superimposed on the data. The first (----) is a plot of the combined product liberation (lysophosphatidylinositol + glycerophosphoinositol), which to a first approximation is a measure of phospholipase A activity. The second (.....) is glycerophosphoinositol production standardized to 25 nmol/30 min of this phospholipase A activity. This line is therefore approximately proportional to lysophospholipase activity. ●, Lower phase (glycerophosphoinositol); O, lysophosphatidylinositol.

suggests that the lysophospholipase activity is probably distinct from the phospholipase A₁ activity. The pH optimum of the phospholipase A₁ appears to be in the region of pH 6, whereas the lysophospholipase activity is maximal at pH 8.5. At the lowest pH studied, the major radioactive product (75%) of the hydrolysis of [2-¹⁴C]oleoylglycerophosphoinositol was lysophosphatidylinositol, indicating that the predominant phospholipase at this pH is a phospholipase A₁ activity. Some labelled fatty acid was also liberated at this low pH; we do not know whether this is partly the result of a phospholipase A₂ or of the action of lysophospholipase on [2-¹⁴C]oleoylglycerophosphoinositol because even at this pH there is some total deacylation of the substrate (Fig. 1).

Functional role of the deacylating enzymes

The phospholipase A₁ activity may be similar to the acid phospholipase A₁ (pH optimum 5.3) secreted by the sheep pancreas (Dawson *et al.*, 1982), especially as the fly salivary gland is responsible for secreting some of the enzymes necessary for digestion (Wigglesworth, 1972). Consequently, we looked for, and found, phosphatidylinositol-deacylating activity in the fluid secretion (saliva) emanating from the glands. The saliva produced during stimulation of six glands for 1 h with 1 μ M-5-hydroxytryptamine was pooled and analysed for enzyme activity. At the end of the period of saliva collection, the glands were homogenized and enzyme activity was measured in the supernatant. The activity in saliva was greater than that remaining in the glands, showing that it is a secreted enzyme.

As choline is an important dietary requirement in Diptera (Bridges, 1972), we examined the specificity of the deacylating activities and found that enzyme activity obtained from either saliva or gland homogenates could deacylate phosphatidylcholine. The enzymes showed no pronounced specificity, though phosphatidylinositol and phosphatidylethanolamine were favoured over phosphatidylcholine. However, observations on specificity employing pure phospholipid substrates are of questionable significance when compared with membranes (Dawson, 1973). Nevertheless, secretion into the saliva of an enzyme that will hydrolyse phosphatidylcholine does give the blowfly a potential source of choline when feeding on material containing this phospholipid. Larvae of the housefly (*Musca domestica*) also contain phospholipid-deacylating activities (Hildebrandt *et al.*, 1971), and these too may be secretory rather than intracellular. The same species also possesses a phosphodiesterase that can liberate free choline from the glycerophosphocholine released (Hildebrandt & Bieber, 1972).

If blowfly salivary glands contain non-specific

Fig. 2. Effect of 5-hydroxytryptamine on the efflux of [³H]inositol and [¹⁴C]ethanolamine from prelabelled cells

Groups of three salivary glands were incubated with 20 μ M-*myo*-[2-³H]inositol or 664 μ M-[2-¹⁴C]ethanolamine for 1 h. The glands were washed and the release of label to the bathing medium was measured at 3 min intervals. 5-Hydroxytryptamine (10 μ M) was added to both groups during the period represented by the solid bar. The release of ³H (●) and ¹⁴C (○) is expressed as c.p.m./min per gland. Similar results were obtained in a further experiment.

deacylating enzymes, then it would be of interest to determine whether these activities are enhanced on stimulation of the gland. When glands are prelabelled with [³H]inositol and subsequently washed, there is a slow efflux of label, which is markedly increased upon stimulation with 5-hydroxytryptamine (Fig. 2), as previously described by Fain & Berridge (1979a). However, in a parallel experiment 5-hydroxytryptamine had no effect on the release of [¹⁴C]ethanolamine (Fig. 2) or [¹⁴C]choline (results not shown). In the experiment shown in Fig. 2, the glands used for ¹⁴C efflux had incorporated 28 pmol of [¹⁴C]ethanolamine, which was considerably more than the 8 pmol of [³H]inositol incorporated by the other glands. In the intact gland, therefore, 5-hydroxytryptamine induced a specific hydrolysis of phosphatidylinositol, presumably by stimulating a phosphoinositol phosphodiesterase, without affecting either phosphatidylethanolamine or phosphatidylcholine. Assuming that the deacylating activities show similar specificities *in vivo* as they do *in vitro*, these observations are consistent with the idea that these activities do not function intracellularly, but are digestive enzymes which are secreted into the saliva.

Effect of 5-hydroxytryptamine on deacylation

Litosch & Fain (1981) have reported a Ca²⁺-independent hydrolysis of phosphatidylinositol in

Fig. 3. *Phosphatidylinositol hydrolysis in homogenates of blowfly glands prelabelled with [3 H]inositol and treated with 5-hydroxytryptamine*

Prelabelling of the glands was as described by Fain & Berridge (1979a). Thirty glands were homogenized in 200 μ l of Tris buffer. Portions of this homogenate were incubated at 30°C. The reaction was stopped by the addition of 0.5 ml of chloroform/methanol (1:2, v/v), and then 1.5 ml of butan-1-ol was added, followed by 1.5 ml of water. The separation and designation of products was as in Fig. 1. Samples of lower (water-soluble) phase were analysed by ionophoresis (see the Materials and methods section), and in both control and 5-hydroxytryptamine-treated samples, glycerophosphoinositol was the major (>95%) water-soluble inositol ester released, though in the zero-time tubes [3 H]inositol was the major radioactive compound. ● and ○, Water-soluble radioactivity; □ and ■, lysophosphatidylinositol; △ and ▲, phosphatidylinositol. Open symbols, controls; closed symbols, +10 μ M 5-hydroxytryptamine. Very similar results were obtained in two similar experiments (see also the text).

homogenates of blowfly salivary glands prelabelled with [3 H]inositol. We carried out some similar experiments, and, like those authors, observed a rapid disappearance of [3 H]phosphatidylinositol when glands were homogenized. However, lysophosphatidylinositol and glycerophosphoinositol were the major products, and we detected virtually no phosphoinositol or cyclic-1:2-phosphoinositol. This hydrolysis was not affected by 10 μ M 5-hydroxytryptamine (Fig. 3), a concentration at which stimulated hydrolysis of phosphatidylinositol in the intact gland is maximal (Fain & Berridge,

1979a). The ratios of c.p.m. in 5-hydroxytryptamine-treated samples to control samples for the data shown in Fig. 3 are 1.144 ± 0.250 (mean $\pm$ s.d.) for water-soluble counts, 1.029 ± 0.041 for lysophosphatidylinositol and 0.958 ± 0.075 for phosphatidylinositol. In an identical experiment (but which included an additional pair of samples at 30 min), the corresponding values were 0.982 ± 0.034 , 1.121 ± 0.142 and 1.000 ± 0.049 respectively. Thus we cannot confirm the reported effect of 5-hydroxytryptamine on gland homogenates, and any hydrolysis is, in our experiments, caused by the deacylating activities described here.

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