THYROTROPIN-STIMULATED PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE A_2 IN PIG THYROID, A RE-EXAMINATION

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Received 21 August 1980

1. Introduction

Haye et al. [1,2] have described previously a phosphatidylinositol-specific phospholipase A_2 in thyroid homogenates which is stimulated by thyrotropin, and have suggested a role for it in the release of arachidonic acid for prostaglandin synthesis. They did not, however, clearly identify all the products formed after PI degradation. The release of free fatty acid from PI can also be effected by the combined action of PI-phosphodiesterase (EC 3.1.4.10) and diacylglycerol lipase; we have suggested that this latter route might be of prime importance in releasing arachidonic acid in vivo in a number of physiological systems [3–5], and experimental evidence that this is so has been obtained in aggregating platelets [6,7].

We have, therefore, attempted to repeat the experiments of Haye et al. [1] using [^{3}H]oleoyl-PI and [^{32}P]PI as substrates, both to confirm the thyrotropin stimulation of fatty acid release, and to identify lyso-PI as a product. We have been unsuccessful in both attempts: we can find no evidence of an enzymic activity hydrolysing PI in thyroid homogenates other than the PI-phosphodiesterase (with or without thyrotropin) and, although the release of free fatty acid from [^{3}H]oleoyl-PI is stimulated by thyrotropin, addition of the BSA + lactose solution in which the hormone is dissolved has an identical stimulatory effect.

2. Materials and methods

Wherever possible the methods followed were those used by Haye et al. [1]. Thyroid glands were taken from pigs killed in this Institute, and homogenized as in [1]. Identical results were obtained from pigs varying from 1 week to 2 years old. Incubations (30 min) were performed as in [1] (except 2 mg of protein per tube was used) with substrates suspended as in [1] at a final concentration of 0.3 mM, and fatty acids were extracted according to Dole [8]. Before counting of the radioactivity in free fatty acid however, the fatty acids were separated from diacylglycerol by TLC in chloroform/methanol/acetic acid (60:50:1 v/v/v); appropriate markers were added and made visible by exposure to iodine before scraping into 1 ml of methanol in scintillation vials to which scintillant was then added.

For extraction and identification of lyso-PI, the extraction method of Bremer was used [9] with lyso-PI carrier (prepared by the method of [10]) to check the yield and carry over, and also as a marker for subsequent separation by TLC in chloroform/methanol/880 ammonia/H_2O (63:35:2:3 by vol.). Water-soluble products released from [^{32}P]PI were separated by ionophoresis [11] with appropriate markers.

[^{32}P]PI was prepared as in Irvine et al. [12] and [^{3}H]oleoyl-PI and [^{3}H]oleoyl-PC as in Irvine and Dawson [5].

Thyrotropin was a gift from the National Institute of Biological Standards, Holly Hill, Hampstead, London, and was dissolved in a serum albumin solution as recommended by that Institute. The 20 mg tablets contained about 19 mg of lactose, and were dissolved in 1% BSA at a concentration of 250 milliunits per ml.

Abbreviations: PI, phosphatidylinositol; PC, phosphatidylcholine; BSA, bovine serum albumin

3. Results and discussion

Table 1 summarizes the results of a typical experiment demonstrating the effect of thyrotropin on the diacylglycerol and free fatty acid release from [^{3}H]oleoyl-PI catalysed by a thyroid homogenate. The enzymic activities we observed were very much lower than those seen by Haye et al. [1]. These authors do not state how much substrate was added to their incubations, but if one assumes a protein phospholipid ratio of approximately 0.5 for a thyroid homogenate, then it is apparent that in their experiments a quantity of substrate (PC or PI) equal to the total thyroid phospholipid added to each tube (about 0.26 μmol) is hydrolysed within 2 min. We did not observe anything within an order of magnitude of this activity with either PC or PI substrates (table 1). We have not, admittedly used thyroid phospholipids as substrates, but as it is clear from Haye et al.'s data [1] that fatty acids other than arachidonate were liberated in considerable amounts by thyroid homogenates, and as oleic acid is a major 2-acyl fatty acid in this tissue [13], it seems unlikely that our use of pure [^{3}H]oleoyl-labelled phospholipids would cause this difference.

Another possible contributory factor may be that pure PI is very difficult to prepare free of phosphatidylserine, phosphatidylethanolamine or phosphatidic acid, all of which are potent stimulators of the PI-phosphodiesterase [14]. For the series of experiments typified by table 1, we added 10% phosphatidic acid and 12% phosphatidylethanolamine to the PI substrate to boost the activity; however, very similar results to table 1 (with an overall lower level of fatty acid and diacylglycerol release) were obtained with >99% pure PI (results not shown). Despite these quantitative discrepancies with the data of Haye et al. [1] it is apparent from table 1 that we have, in principle at least, confirmed their results; a 10-fold stimulation of free fatty acid release is caused by thyrotropin with no such stimulation for PC (data not shown). Table 1 also includes data for tubes to which the 1% BSA, 0.3% lactose solution in which the thyrotropin is resuspended was added, and it is apparent that the effect of this solution is identical to that of thyrotropin.

Table 1
Products released from oleoyl PI by a thyroid homogenate in 30 minutes

	Free fatty acid (nmole)	Diacylglycerol (nmole)
Control	0.88	2.46
	0.44	2.94
+ Thyrotropin	6.45	8.62
	6.25	9.79
+ BSA + lactose	6.25	7.30
	6.20	6.61

Data show duplicate readings from one experiment. Similar results were obtained in identical experiment. 12 000 dpm of oleoyl PI were added to each tube. Data are expressed as net release of products over tubes in which the homogenate and substrate were incubated separately

Parallel experiments (with both pure PI and with PI + phosphatidic acid and phosphatidylethanolamine) were performed using ^{32}P-labelled PI as a substrate, and we measured the radioactivity in lyso-PI and water-soluble inositol esters (no radioactive inorganic phosphate was released). We could detect no accumulation of radioactivity in lyso-PI with or without thyrotropin, and the only water-soluble compound released from PI was phosphoinositol; the TCA precipitation technique [15] does not permit the separation of phosphoinositol from the cyclic form [16]. No glycero-phosphoinositol release could be detected even in the presence of a 2 mM glycerophosphoinositol 'trap' which, it could be clearly seen, was not hydrolysed during the incubation. The release of phosphoinositol was stimulated by the addition of thyrotropin (or BSA alone). Thus we were unable to establish any evidence for deacylation of PI with or without thyrotropin, and can only assume that the free fatty acid released (table 1) must have come from the combined action of PI-phosphodiesterase and diacylglycerol lipase.

A stimulation of PI-phosphodiesterase and diacylglycerol lipase by BSA could be caused by a variety of factors. The latter enzyme is apparently stimulated to a greater extent (table 1), which may reflect its higher requirement for calcium; addition of calcium (1 mM) to the tubes reduced (but did not completely remove) the BSA stimulation. We observed also that the degree of stimulation by BSA depended on the dilution of thyroid homogenate in the final incubation: the more dilute the homogenate, the greater the stimulation. This, allied with a clear non-linear increase in enzyme activity with increasing homogenate concentration, suggests that BSA is to some extent supplying an intrinsic factor (or factors) found in the homogenate. Free fatty acid, another

component of BSA [17] is also stimulatory to the PI-phosphodiesterase [14]; commercial preparations of BSA vary considerably in their fatty acid content [17] and in fact we found a considerable variation in the stimulating effect caused by a series of different commercial BSA preparations. Some were indeed inhibitors, perhaps a reflection of their differing content of lipoprotein, a potent inhibitor of the PI-phosphodiesterase [18]. We found no evidence in our BSA samples of an intrinsic phospholipase activity [19].

Finally, it is relevant to add here that Haye and Jacquemin [20] have also reported a stimulatory effect of thyrotropin on the PI-phosphodiesterase activity of particle-free thyroid supernatants (assayed at pH 5.3 with 0.4 μM Ca^{2+}). We have been unable to reproduce this stimulation. Some BSA samples (with or without thyrotropin) gave a stimulation with no added calcium, but none had any effect (or were inhibitory) in the presence of calcium.

In summary, we have not been able to confirm exactly quantitatively the results of Haye et al. [1]. Although we discuss above several possible contributory factors, it is still difficult to account for so large a discrepancy in the enzyme activities observed – especially with PC as a substrate where many of these factors do not apply. We have, however, qualitatively confirmed a stimulated release of free fatty acid from PI caused by the addition of thyrotropin solutions. In our hands this does not appear to be an event mediated by thyrotropin through its hormonal receptors, nor one caused by a stimulation of a phospholipase A_2 activity.

Acknowledgements

We thank Norma Hemington for preparing the [^{32}P]PI, and the National Institute of Biological Standards for the gift of thyrotropin.

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