Regulation of myo-inositol transport during the growth and differentiation of thyocytes: a link with thyroid-stimulating hormone-induced phospholipase A$_2$ activity

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The $V_{max}$ of myo-inositol transport increased 3-fold during epidermal growth factor (EGF)-induced growth and thyroid-stimulating hormone (TSH)-induced differentiation in primary cultures of sheep and human thyrocytes. The $K_m$ remained unaltered. This up-regulation required the presence of insulin. The TSH-induced rise in myo-inositol transport commenced 8 to 16 h after the initial stimulus and achieved a plateau at 24 h. In human thyrocytes the change in $V_{max}$ was accompanied by an increase in the steady-state levels of mRNA for the myo-inositol transporter following treatment with either ligand. Examination of the metabolites of myo-inositol showed few significant changes after treatment of sheep thyrocytes with EGF for 24 h. This is consistent with maintenance of the intracellular concentration of myo-inositol as the cells enlarge in preparation for cell division. In TSH-treated cells, however, up-regulation of myo-inositol transport was linked with increased myo-inositol cycling across the cell membrane, increased phospholipase A$_2$-mediated turnover of phosphatidylinositol and a concomitant increase in arachidonic acid turnover. Increased levels of myo-inositol phosphates were also noted 24 h after TSH treatment. These results indicate the initiation of secondary signalling events many hours after the primary stimulus.

INTRODUCTION

Although epidermal growth factor (EGF) and thyroid-stimulating hormone (TSH) both stimulate thyroid growth, their effects on thyroid function are antagonistic [1,2]. EGF inhibits thyroid iodide uptake, prothyroid hormone synthesis, thyroperoxidase synthesis and iodide organisation, all of which are required for thyroid hormone synthesis [3]. The signalling events mediated by these ligands in fully differentiated thyroid cells, and the interactions between the signalling pathways, remain ill-defined. The TSH receptor is coupled to adenylate cyclase activation and many of the effects of TSH on thyroid function are mimicked by forskolin and dibutyryl cyclic AMP (dbcAMP). However, transfection of the cloned human TSH receptor into Chinese hamster ovary cells has shown that the receptor can also be coupled to second messengers generated following inositol lipid hydrolysis [4]. This coupling is presumably through G$_i$ and phospholipase C$_{IB}$ (PLC$_{IB}$) activation. Whether TSH activates this pathway in thyroid cells from all species is a matter of controversy which may be resolved by the findings of regulation of TSH receptor signalling by purinergic receptors. Adenosine has been shown to sensitize the TSH-coupled PLC pathway in FRTL5 cells [5], a rat thyroid-derived cell line which shows some differentiated thyroid function. In this cell line, antibodies to the TSH receptor have been shown to activate phospholipase A$_2$ (PLA$_2$) [6] and, consistent with this, many earlier studies have shown TSH-induced increases in arachidonate and prostaglandins [7–11]. The actions of TSH in stimulating thyroid cell growth and differentiation require the presence of insulin or insulin-like growth factor 1 (IGF-1) [12–14], implying that cooperation is required with a second signalling pathway. Thus TSH clearly utilizes a range of signalling mechanisms.

A similar conclusion can be made with regard to EGF-induced signalling. The EGF receptor has intrinsic tyrosine kinase activity which is revealed upon EGF binding. This receptor has recently been shown to activate PLC$_{y1}$ through phosphorylation of its SH2 domain [15]. Consistent with activation of this pathway, which would result in protein kinase C (PKC) activation, the phorbol ester phorbol 12-myristate 13-acetate can mimic many of the EGF effects in the thyroid and elsewhere [16]. EGF can inhibit many aspects of TSH-induced thyroid cell differentiation in functional primary cell cultures [1,2,17,18]. In vitro-transformed FRTL5 cells, activation of PKC inhibited the migration of the catalytic subunit of protein kinase A (PKA) to the nucleus, illustrating the possible basis for interactions between the signalling pathways [19]. At the cell membrane, however, there is no evidence for interference by EGF in the response of thyroid cells to acute challenge with TSH in cyclic AMP assays, although accumulated levels of cyclic AMP were reduced in EGF plus TSH-treated cells [16].

There is no reason to suppose that all of the signalling pathways involved in either TSH or EGF actions are known. Indeed evidence exists that there may be more pathways awaiting discovery. One long-standing unexplained observation is that of Scott et al. [20], who demonstrated a TSH-stimulated increase in de novo phosphatidylinositol (PtdIns) synthesis in sheep and dog thyroid slices. In their study, the amount of PtdIns synthesis was well in excess of that which would be required to resynthesize PtdIns(4,5)P$_2$ after PLC-mediated hydrolysis.

Metabolism of myo-inositol is central to cell signalling pathways, e.g. PtdIns(4,5)P$_2$ hydrolysis and receptor-mediated PtdIns(3,4,5)P$_3$ synthesis [21]. However, these pathways consume only a small fraction ($\approx 5\%$) of the total myo-inositol present in cells. myo-Inositol is present in many cells at high concentrations

Abbreviations used: ANOVA, analysis of variance; dbcAMP, dibutyryl cyclic AMP; EGF, epidermal growth factor; GroPLns, glycerophosphoinositol; IGF-1, insulin-like growth factor 1; PKA, cyclic AMP-dependent protein kinase A; PKC, protein kinase C; PLA$_2$, phospholipase A$_2$; PLC, phospholipase C; PtdIns, phosphatidylinositol; TEAB, trimethylammonium bicarbonate; TSH, thyroid-stimulating hormone; inositol phosphates are described according to Biochem. J. (1989) 258, 1–2.

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as free myo-inositol, myo-inositol phosphates, PtdIns and its phosphorylated derivatives, and inositol glycans (reviewed in [22,23]). In most cell types the intracellular myo-inositol concentration is regulated by the rate of myo-inositol transport [24], and the transporter itself is regulated during differentiation [24,25]. There is therefore potential for greater involvement of myo-inositol in signalling pathways than is presently appreciated.

Using fully differentiated sheep and human thyroid cell cultures we have examined myo-inositol transport and its subsequent metabolism following treatment of the cells with TSH and EGF. These cells have advantages over studies with the rat thyroid cell line (FRTL5) or studies with primary dog thyroid cultures because they maintain the synthesis of thyroid hormones de novo [3].

**EXPERIMENTAL**

**Cell culture**

The isolation of sheep thyroid cells and their serum-free culture were as previously described [12]. Cells were used 7 to 10 days after isolation when a confluent monolayer had formed. Cultures were incubated in the absence of TSH or insulin for days (two medium changes) prior to the start of the experiment. Following these pre-incubation conditions TSH did not stimulate thyroid cell growth to any appreciable extent [2]. Human thyroid tissue was obtained from the Queen Elizabeth Hospital, Birmingham and cells were isolated and cultured as described for sheep thyroid. The thyroid samples were of histologically normal tissue as determined by the hospital Pathology Department.

**Assessment of myo-inositol synthesis**

To determine whether thyrocytes synthesized myo-inositol from glucose, cultures were labelled with 41 μCi/10⁶ cells of D-[U-14C]-glucose (292 mCi/mmol, Amersham International, Aylesbury, U.K.) for 24 h. Total lipid extracts were isolated from the labelled cells and chemically degraded to yield samples of their fatty acid, glycerol and myo-inositol components, for all of which glucose is the only, or a major, biosynthetic precursor [26]. These moieties were separated by paper chromatography and their identity confirmed by ion-moderated partition chromatography on an Aminex HPX-87C carbohydrate column [26].

**myo-inositol transport assay**

For assays of myo-inositol transport, cultures were washed three times with 1 ml of incubation buffer (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 0.8 mM MgCl₂, pH 7.4). Buffer (500 μl) containing 0.1–1.0 μCi of inulin-[14C]-carboxyl (1–3 μCi/mg, ICN Flow, High Wycombe, Bucks., U.K.) was added. After 10 min incubation at 37 °C, 500 μl of incubation buffer containing myo-inositol (5–100 μM) and 0.2–4.0 μCi/mmole of myo-[2-3H]-inositol (10–20 Ci/mmole, Amersham International, Aylesbury, Bucks., U.K.) were added. The cultures were incubated for a further 10 min at 37 °C. The assay was stopped by aspiration of the medium. The cells were washed three times in incubation buffer and the cell layer dissolved in 200 μl of 0.1 M NaOH. The lysates were transferred to scintillation vials containing scintillation fluid and the radioactivity determined. Kinetic parameters were determined from Hanes plots of the data.

**Extraction of myo-inositol and its derivatives**

To determine the steady-state levels of myo-inositol and its derivatives, cultures were equilibrium labelled (4 days) with myo-[2-3H]-inositol (2 μCi/ml) in NCTC135:mF12M (serum-free) medium in the ratio 7:5 (v/v) (final myo-inositol concentration of 42 μM). To determine the non-equilibrium distribution of myo-inositol, unlabelled cultures were exposed to pre-warmed incubation buffer containing 50 μM myo-inositol and myo-[2-3H]-inositol (0.58 μCi/nmol) for 15 min at 36 °C. The cells were either washed and extracted (pulse-labelled cells), or the buffer was removed and the cells were further exposed to incubation buffer containing 50 μM myo-inositol for 15 min at 37 °C before extraction (pulse-chase-labelled cells). To determine the efflux of myo-inositol from cells to the medium, cultures were non-equilibrium labelled for 15 min as above, were quickly washed three times with a 5-fold excess of incubation buffer, and were then incubated for 15 min with incubation buffer containing 50 μM unlabelled myo-inositol only. The buffer was collected and the radioactivity determined.

In equilibrium experiments amounts of soluble myo-inositol metabolites in cells were presented as μM averaged throughout the cell volume. For non-equilibrium experiments data were expressed as d.p.m./10⁶ cells per fl. The specific activity of labelling was not the same for the equilibrium and non-equilibrium experiments; therefore direct comparisons of d.p.m./10⁶ cells per fl would not be valid. To allow for comparison of data the non-equilibrium data were also presented as μM averaged throughout the cell volume. It should be pointed out therefore that these are not true concentrations. These procedures were necessary to correct for the large volume changes found upon treatment of the cells with the various hormones. myo-Inositol recycled to the medium was expressed as pmol/10⁶ cells.

In all cases myo-inositol and its derivatives were extracted according to the method of Wregget and Irvine [27]. The lipid fraction was deacylated according to Clarke and Dawson [28]. The soluble and deacylated lipid fractions were separated by ion-exchange chromatography on Sep-Pak QMA columns (Waters, Maidstone, U.K.) using a modification of the method of Maslanski and Bua [29]. Specifically, extracts were first brought to a pH greater than 9.0 by the addition of a few drops of 1 M NaOH. myo-Inositol was eluted in 15 ml of water, glycerophosphoinositol (GroPIns), InsP₁, InsP₂, InsP₃, InsP₄, and InsP₅ were eluted with 5 ml each of 0.01 M, 0.10 M, 0.25 M, 0.325 M, 0.40 M, 0.50 M, and 0.60 M triethylammonium bicarbonate (TEAB) respectively. For separation of deacylated lipids GroPIns, GroPInsP₁, and GroPInsP₂ were eluted with 5 ml each of 0.10 M, 0.25 M, and 0.325 M TEAB respectively. Separation of all fractions was monitored by the addition of myo-[U-14C]-inositol (304 mCi/mmol; Amersham International, Aylesbury, U.K.) labelled standards prepared according to French et al. [26]. Typical separations are shown in Figure 1.

**Arachidonic acid extractions**

To determine the steady-state levels of arachidonic acid, cultures were equilibrium labelled (4 days) with 0.3 μCi/ml of [1-14C]acetic acid, sodium salt (56 mCi/mmol, Amersham International, Aylesbury, U.K.). Where specified, indomethacin (Sigma, Poole, U.K.) was added to a final concentration of 15 μM for the last 2 h of culture. To determine the non-equilibrium levels of arachidonic acid, cultures were labelled for 2 h with 2.5 μCi/10⁶ cells of [1-14C]acetic acid (sodium salt) in the presence of 15 μM indomethacin. Eicosanoids were extracted according to McDonald-Gibson [30] and were separated by TLC using the A9 solvent system, employing the following standards: arachidonic acid, prostaglandin F₂α, 6-keto-prostaglandin F₁α, prostaglandin D₂, prostaglandin E₅ (15S)-hydroperoxy-(5Z,8Z,11Z,13E)-eicosatetraenoic acid, and (5S,15S)-dihydroxy-(6E,8Z,11Z,13E)-
eicosatetraenoic acid (all from Sigma) made up to 1 mg/ml in ethyl acetate [31]. Location of radioactivity on the plates was determined by autoradiography and the appropriate spots were scraped into scintillation fluid and the radioactivity assayed. To account for differences in loading of the lanes, results were calculated as the ratio of d.p.m. in the arachidonic acid spot to d.p.m. in the phospholipid (origin) spot [31].

Cell volume determinations
Cell volumes were determined by indirect immunofluorescence staining using a primary rabbit anti-PKCζ antibody (Gibco, Uxbridge, U.K.) and a secondary fluorescein-conjugated sheep anti-rabbit IgG antibody (Gibco, Uxbridge, U.K.). Cells were counterstained with propidium iodide. Cell surface areas and volumes were determined by laser scanning confocal microscopy (Bio-Rad MRC 500 laser scanning confocal microscope) at 400 times magnification using 0.6 μm scans in the x–y and x–z sections. It was previously determined that treatment of the cells with EGF did not induce significant rearrangement in the subcellular localization of PKCζ, while TSH treatment induced a rearrangement of PKCζ to the cytoskeleton, and thus the limits of the cell were still defined [32].

Northern-blot analysis
For Northern-blot analysis, total RNA was extracted according to the method of Chomczynski and Saachi [33], and was separated on a 1.0% agarose gel in 2.2 M formaldehyde. Blots of the gel were probed with random-primer-labelled cDNA to canine kidney Na+/myo-inositol co-transporter (a kind gift from Dr. M. Kwon, Johns Hopkins University [34]) using the procedures described by Church and Gilbert [35].

Statistical analyses
Statistical analysis of the results was by analysis of variance (ANOVA). Data are presented as the mean ± S.E.M.

RESULTS
Assessment of myo-inositol synthesis by thyrocytes
The ability of sheep thyrocytes to synthesize myo-inositol was determined in cultures which had been treated with insulin (1 μg/ml) alone or insulin plus TSH (0.3 nM) for 24 h in the presence of [14C]glucose. The total lipid fraction was isolated and chemically degraded to yield the glycerol and myo-inositol components for which glucose is the only, or a major, biosynthetic precursor. The glycerol moieties were effectively labelled by 14C.
Figure 3 Characteristics of myo-inositol transport in sheep thyrocytes

myo-inositol transport in sheep thyrocytes was assayed as detailed in the Experimental section. The Figure shows the rate of myo-inositol transport (pmol/min per 10⁶ cells) with respect to substrate concentration (µM). A Hanes plot of the data is inset. All data points represent the mean ± S.E.M. of 12 separate experiments.

derived from glucose (insulin-treated cells, 4407 ± 308 d.p.m./10⁶ cells; insulin + TSH-treated cells, 7555 ± 680 d.p.m./10⁶ cells; mean ± S.E.M., n = 3) but the majority of the myo-inositol remained unlabelled (insulin-treated cells, 58 ± 5 d.p.m./10⁶ cells; insulin + TSH-treated cells, 200 ± 18 d.p.m./10⁶ cells; mean ± S.E.M., n = 3) (Figure 2). It was estimated that under the conditions of culture used, thyrocytes derived the majority (87%) in TSH-stimulated cells and 93% in control cells) of their myo-inositol from the extracellular medium. The low levels of endogenous myo-inositol synthesis thus allowed the accurate measurement of myo-inositol transport and of the intracellular levels of myo-inositol and its derivatives.

Characteristics of the myo-inositol transporter

myo-Inositol transport in sheep thyrocytes was linear with respect to cell number over the range of (0.4–1.0) × 10⁶ cells and with respect to time over at least 10 min, suggesting that the time course examined represented initial rates of accumulation. Data presented in Table 3 show rapid cycling of myo-inositol between the intracellular and extracellular compartments with an influx rate exceeding the efflux rate by approximately 2-fold. The measured myo-inositol transport rate represents the accumulation of myo-inositol over and above the efflux rate, and as such is probably an underestimate of the true transport rate. The prolonged linearity of myo-inositol uptake is a characteristic of myo-inositol transport reported in a number of cell types (e.g. [36,37]). A plot of uptake rate versus substrate concentration showed that at higher substrate concentrations, transport was saturable (Figure 3). Kinetic analysis of the data (Figure 3, inset) yielded a Km of 41.8 ± 4.5 µM (n = 12) and a Vmax of 4.5 ± 0.29 pmol/min per 10⁶ cells (n = 12). These values closely match the kinetic parameters of myo-inositol transport from a wide variety of tissues and species [24,36].

myo-Inositol transport in all cell types so far examined is sodium dependent [38]. Transport in sheep thyroid cells was assayed at a substrate concentration of 25 µM in the presence or absence of sodium (140 mM) in the incubation buffer. Where sodium was omitted it was replaced with 140 mM CsCl. At 140 mM sodium the transport rate was 1.04 ± 0.07 pmol/min per 10⁶ cells (n = 3), while at 0 mM sodium the rate was 0.08 ± 0.007

(n = 3; P < 0.001) pmol/min per 10⁶ cells. Therefore in 25 µM myo-inositol 92% of transport is sodium-dependent.

The allosteric effect of Mg²⁺ on myo-inositol binding to the sheep thyrocyte transporter [39] was examined. In the absence of Mg²⁺ the Km was 82.2 ± 15.6 µM (n = 8) which fell to 42.8 ± 4.5 µM at 0.8 mM Mg²⁺ (n = 12; P < 0.01). This represented a doubling in the affinity of the transporter for its substrate in the presence of Mg²⁺. The Vmax was unaffected by Mg²⁺.

TSH, Insulin, and EGF effects on myo-inositol transport

The effect of TSH concentration on the Vmax for myo-inositol transport, following treatment for 24 h, is shown in Figure 4(a). The effect was maximal at 0.3 nM TSH (the value obtained for 3.0 nM TSH was not significantly different from that at 0.3 nM). High TSH concentrations (10 nM) produced a fall in Vmax from the plateau value to 8.13 ± 2.0 pmol/min per 10⁶ cells (n = 3; P < 0.05). An inhibition of differentiated function at high doses of TSH has previously been reported for other TSH-induced functions [18,32]. Thus the dose response for the increase in Vmax by TSH parallels that for the induction of differentiated function. There was no effect on the Km across the dose range. The effects of TSH on the Vmax of myo-inositol transport were reproduced by 1 mM dbcAMP, which induced a rise in Vmax from 4.05 ± 0.29 pmol/min per 10⁶ cells (n = 12) to 21.96 ± 2.36 pmol/min per 10⁶ cells (n = 3; P < 0.005).
There was a low rate of myo-inositol transport in the absence of insulin ($V_{max}$, of $1.58 \pm 0.46$ pmol/min per $10^6$ cells; $n = 5$) which rose with insulin dose to reach $4.05 \pm 0.29$ pmol/min per $10^6$ cells ($n = 12$) in the presence of $1 \mu$g/ml of insulin ($P < 0.01$). There was no change in $K_m$ at the insulin doses used in this experiment. In the absence of insulin the $V_{max}$ was unaltered by concentrations of TSH up to $3.3$ nM (Figure 4a).

The effects of EGF treatment for $24$ h in the presence of insulin ($1 \mu$g/ml) are shown in Figure 4(b). EGF increased the $V_{max}$ without affecting the $K_m$ of myo-inositol transport. The magnitude of the effect was dependent on EGF concentration and was optimal at $1 \times 10^{-8}$ M.

Optimal doses of TSH ($0.3$ nM) and EGF ($1 \times 10^{-8}$ M) were simultaneously given to the cells for $24$ h in the presence of insulin ($1 \mu$g/ml). This treatment produced a $V_{max}$ of $9.62 \pm 0.82$ pmol/min per $10^6$ cells ($n = 5$), which was not significantly different from the $V_{max}$ obtained in the presence of TSH or EGF alone.

**Time course of the effect of TSH on myo-inositol transport**

Sheep thyrocytes were cultured in the presence of insulin ($1 \mu$g/ml) and were treated with $0.3$ nM TSH for various time periods (Figure 5). There was no acute effect of TSH when examined after $10$ min or $8$ h. However, at $16$ h the $V_{max}$ was significantly elevated over control values and continued to rise until a plateau was achieved at $24$ h. There were no further changes in $V_{max}$ up to the longest time point examined ($96$ h), nor was there any significant alteration in $K_m$ across the time course. The rise in $V_{max}$ paralleled the time course for acquisition of other differentiated characteristics such as the ability to trap iodine and to synthesize thyroglobulin [18,40].

**myo-Inositol transport in human thyrocytes**

Human thyrocytes were cultured in the presence of $1 \mu$g/ml insulin and were exposed to $0.3$ nM TSH or $1 \times 10^{-8}$ M EGF for $24$ h. In control cells (insulin alone) the $K_m$ ($29.9 \pm 4.3 \mu$M; $n = 3$) and $V_{max}$ ($4.10 \pm 0.71$ pmol/min per $10^6$ cells; $n = 3$) were not significantly different from the values obtained with sheep thyrocytes. TSH treatment for $24$ h produced a rise in $V_{max}$ to $24.60 \pm 2.18$ pmol/min per $10^6$ cells ($n = 3$) with no significant change in $K_m$. EGF treatment for $24$ h produced a rise in $V_{max}$ to $10.35 \pm 1.24$ pmol/min per $10^6$ cells ($n = 3$) also with no significant change in $K_m$.

**Regulation of human thyroid myo-inositol transporter mRNA**

To examine transcriptional regulation, total RNA extracted from human thyrocytes treated with TSH, EGF, or a combination, was analysed on a $1.0\%$ agarose gel in formaldehyde. A canine kidney Na+/myo-inositol co-transporter cDNA [34] was used to probe a blot of the gel (Figure 6; representative of three separate blots). A photograph of the ethidium bromide-stained gel before blotting shows that all lanes were equally loaded. No cross-hybridization of the probe with sheep RNA was observed (data not shown). Hybridization with a band of $10.5$ kb human mRNA was observed. The levels of this mRNA were increased by treatment of human thyroid cells with TSH or EGF, indicating that the increases in $V_{max}$ seen were due to increased steady-state levels of mRNA.

**Effects of TSH, EGF and TSH + EGF on myo-inositol metabolism: equilibrium labelling study**

To determine the consequences of the up-regulation of myo-inositol transport, the fate of transported myo-inositol in response to TSH or EGF treatment of sheep thyrocytes was determined. Thyrocytes were labelled to equilibrium (4 days), treated for $24$ h with the appropriate ligand, and myo-inositol and its derivatives were extracted (Table 1). EGF treatment resulted in a significant rise in the level of PtdIns$_{P2}$ from $11.0 \pm 0.99$ pmol/10$^6$ cells to $18.32 \pm 2.09$ pmol/10$^6$ cells ($P < 0.025$). Elevations in Ins$_{(1,4,5)}$P$_3$ can last for several hours after receptor activation [41] and the increase in PtdIns$_{P2}$ may be related to the regeneration of this lipid after the termination of the PLC$_Y_1$-mediated hydrolysis. There was no significant change in concentration of myo-inositol or any of its soluble derivatives with the exception of Ins$_{P2}$, which showed a small but significant rise from $0.72 \pm 0.11 \mu$M to $1.09 \pm 0.12 \mu$M ($P < 0.05$).
Table 1  Equilibrium levels of inositol metabolites in sheep thyrocytes
Sheep thyrocytes were equilibrium labelled with [3H]myo-inositol and were treated with either TSH (0.3 nM), EGF (1 x 10⁻⁸ M), TSH plus EGF (0.3 nM plus 1 x 10⁻⁸ M), or dbcAMP (1 mM) for 24 h in the presence of insulin (1 μg/ml). Inositol metabolites were extracted and analysed as described in the Experimental section. The concentrations of soluble inositol metabolites are expressed as μM averaged throughout the cell volume. (Insulin-treated cells had an average volume of 2025 μm³; this rose to 2902 μm³ after 24 h of EGF treatment, and fell to 896 μm³ after 24 h of TSH treatment.) The concentrations of inositol lipids are expressed as pmol/10⁶ cells. Data points represent the mean ± S.E.M. of 3–14 separate experiments. Values marked with an asterisk are significantly different from the corresponding value in insulin-alone-treated cells at least P < 0.05.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Treatment…</th>
<th>Insulin</th>
<th>Insulin + TSH</th>
<th>Insulin + EGF</th>
<th>Insulin + TSH + EGF</th>
<th>Insulin + dbcAMP</th>
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<tr>
<td>myo-inositol</td>
<td>1635 ± 205</td>
<td>2661 ± 446*</td>
<td>1824 ± 278</td>
<td>2960 ± 398*</td>
<td>5630 ± 58*</td>
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<tr>
<td>GroPins</td>
<td>21.14 ± 4.3</td>
<td>29.70 ± 7.19</td>
<td>19.15 ± 3.27</td>
<td>65.45 ± 4.49*</td>
<td>550 ± 5.6</td>
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<td>InsP</td>
<td>1.67 ± 0.26</td>
<td>2.72 ± 0.79</td>
<td>2.39 ± 0.35</td>
<td>3.99 ± 0.61*</td>
<td>19.39 ± 4.17*</td>
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<td>InsP₂</td>
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<td>1.16 ± 0.11</td>
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<td>0.71 ± 0.15*</td>
<td>0.30 ± 0.04</td>
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<td>0.92 ± 0.23</td>
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<td>InsP₅</td>
<td>2.30 ± 0.22</td>
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<td>InsP₆</td>
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<td>1.66 ± 0.399</td>
<td>1.09 ± 0.12*</td>
<td>2.36 ± 0.26*</td>
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<tr>
<td>Ptdlns</td>
<td>383.3 ± 31</td>
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<td>434 ± 25</td>
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<td>20.9 ± 1.6</td>
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TSH treatment of the thyrocytes produced a markedly different result. There was a 1.6-fold increase in the intracellular concentration of myo-inositol (P < 0.025) accompanied by significant rises in the concentrations of the soluble inositol phosphates, InsP₃, InsP₄, InsP₅ and PtdIns (Table 1). The rise in intracellular myo-inositol concentration is in accordance with the rise in myo-inositol transport Vₘₐₓ seen in TSH-treated thyrocytes. The rise in the levels of InsP₃, InsP₄ and InsP₅ is consistent with a PtdIns₄ hydrolysis signalling event. Changes in intracellular concentrations of InsP₃ and InsP₄ have been previously noted in HL60 cells and normal blast cells undergoing differentiation [26,42,43]. DbcAMP was able to reproduce many of the effects of TSH as shown in Table 1. The relative differences in the levels of InsP and InsP₄ probably reflect increased flux down these pathways in dbcAMP-treated cells, in accordance with the greater increase in myo-inositol transport Vₘₐₓ.

Treatment of thyrocytes with TSH plus EGF resulted in a significant rise in the levels of myo-inositol and all of its soluble derivatives and also in the levels of PtdInsP. One major difference from that seen with either ligand alone was a noticeable rise in the levels of GroPins from 21.14 ± 4.30 μM to 65.45 ± 4.49 μM (P < 0.01). This molecule reflects PtdIns deacylation by PLA₂ and a lysophospholipase [44].

Effects of TSH, EGF and TSH plus EGF on myo-inositol metabolism: pulse labelling study
Maximal changes in the up-regulation of myo-inositol transport occurred after 24 h of treatment with ligand. The immediate fate of the transported myo-inositol at this time point was therefore determined by pulse labelling of thyrocytes after incubation for 24 h in the presence of agonist (Table 2). No significant amounts of label were found in InsP₃, InsP₄, InsP₅, or InsP₆ under any of

Table 2  Pulse labelling (15 min) of inositol metabolites in sheep thyrocytes
Thyrocytes were incubated with TSH (0.3 nM), EGF (1 x 10⁻⁸ M), or TSH plus EGF (0.3 nM plus 1 x 10⁻⁸ M) for 24 h in the presence of insulin (1 μg/ml). Cultures were pulse-labelled with [3H]myo-inositol for 15 min and the various inositol metabolites extracted as described in the Experimental section. The levels of soluble inositol metabolites are expressed as d.p.m./10⁶ cells per ml averaged throughout the cell volume. Values in parentheses are the same data presented as μM averaged throughout the cell volume as described in the Experimental section. The concentrations of inositol lipids are expressed as pmol/10⁶ cells. Data points represent the mean ± S.E.M. of 3–5 separate experiments. Values marked with an asterisk are significantly different from the corresponding value in insulin-alone-treated cells at least P < 0.05.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Treatment…</th>
<th>Insulin</th>
<th>Insulin + TSH</th>
<th>Insulin + EGF</th>
<th>Insulin + TSH + EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-inositol</td>
<td>19.078 ± 2540</td>
<td>153.467 ± 19.368*</td>
<td>33.835 ± 5096*</td>
<td>87.443 ± 10.958*</td>
<td></td>
</tr>
<tr>
<td>(14.87 ± 1.98)</td>
<td>(119.59 ± 15.09*)</td>
<td>(26.36 ± 3.67*)</td>
<td>(68.41 ± 6.15*)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GroPins</td>
<td>259.8 ± 125.0</td>
<td>1330.2 ± 483.0</td>
<td>406.1 ± 113.6</td>
<td>3271.2 ± 905.2*</td>
<td></td>
</tr>
<tr>
<td>(0.20 ± 0.10)</td>
<td>(1.04 ± 0.38)</td>
<td>(0.32 ± 0.09)</td>
<td>(2.55 ± 0.71)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>InsP</td>
<td>15.99 ± 3.10</td>
<td>71.06 ± 15.72*</td>
<td>21.8 ± 7.36</td>
<td>15.53 ± 1.44</td>
<td></td>
</tr>
<tr>
<td>(0.01 ± 0.00)</td>
<td>(0.05 ± 0.01*)</td>
<td>(0.02 ± 0.01)</td>
<td>(0.01 ± 0.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>InsP₂</td>
<td>9.33 ± 0.48</td>
<td>49.05 ± 8.75*</td>
<td>6.42 ± 0.85</td>
<td>16.02 ± 1.81</td>
<td></td>
</tr>
<tr>
<td>(0.01 ± 0.00)</td>
<td>(0.04 ± 0.01*)</td>
<td>(0.01 ± 0.00)</td>
<td>(0.01 ± 0.00)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ptdlns</td>
<td>0.1006 ± 0.0195</td>
<td>0.4113 ± 0.0404*</td>
<td>0.2301 ± 0.0729*</td>
<td>0.3097 ± 0.0277*</td>
<td></td>
</tr>
<tr>
<td>PtdlnsP</td>
<td>0.0075 ± 0.0041</td>
<td>0.0747 ± 0.0354</td>
<td>0.0189 ± 0.0087</td>
<td>0.0143 ± 0.0015</td>
<td></td>
</tr>
<tr>
<td>PtdlnsP₂</td>
<td>0.0066 ± 0.0034</td>
<td>0.0430 ± 0.0265</td>
<td>0.0342 ± 0.0197</td>
<td>0.0124 ± 0.0036</td>
<td></td>
</tr>
</tbody>
</table>
Table 3  Pulse–chase labelling of inositol metabolites in sheep thyrocytes

Thyrocytes were incubated with TSH (0.3 nM), EGF (1 × 10⁻⁸ M), or TSH plus EGF (0.3 nM plus 1 × 10⁻⁹ M) for 24 h in the presence of insulin (1 µg/ml). Cultures were pulse-labelled with [³H]myo-inositol for 15 min followed by a chase for 15 min with unlabelled myo-inositol. Inositol metabolites were extracted as described in the Experimental section. The levels of soluble inositol metabolites are expressed as d.p.m./10⁶ cells per fl averaged throughout the cell volume. Values in parentheses are the same data presented as µM averaged throughout the cell volume as described in the Experimental section. The concentrations of inositol lipids are expressed as pmol/10⁶ cells. The amount of myo-inositol recycled to the extracellular medium is expressed as pmol/10⁶ cells. Data points represent the mean ± S.E.M. of 3–5 separate experiments. Values marked with an asterisk are significantly different from the corresponding pulse-labelled cells (Table 2) at least P < 0.05.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Treatment . . .</th>
<th>Insulin</th>
<th>Insulin + TSH</th>
<th>Insulin + EGF</th>
<th>Insulin + TSH + EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>myo-Inositol</td>
<td>2304.5 ± 1063.0</td>
<td>4604.6 ± 1708.9</td>
<td>1930.3 ± 583.2</td>
<td>5401.4 ± 2038.2</td>
<td></td>
</tr>
<tr>
<td>GroPIns</td>
<td>26.68 ± 3.31</td>
<td>74.15 ± 8.41</td>
<td>19.90 ± 1.24</td>
<td>96.15 ± 12.79</td>
<td></td>
</tr>
<tr>
<td>InsP</td>
<td>0.02 ± 0.003</td>
<td>0.055 ± 0.0075</td>
<td>0.014 ± 0.002</td>
<td>0.076 ± 0.017</td>
<td></td>
</tr>
<tr>
<td>InsP₂</td>
<td>0.028 ± 0.004*</td>
<td>0.072 ± 0.018</td>
<td>0.036 ± 0.015</td>
<td>0.048 ± 0.005*</td>
<td></td>
</tr>
<tr>
<td>PtdIns</td>
<td>8.46 ± 0.83</td>
<td>16.53 ± 1.32</td>
<td>5.36 ± 0.42</td>
<td>16.91 ± 1.30</td>
<td></td>
</tr>
<tr>
<td>PtdInsP</td>
<td>0.010 ± 0.001</td>
<td>0.03 ± 0.016</td>
<td>0.002 ± 0.0019</td>
<td>0.012 ± 0.0019</td>
<td></td>
</tr>
<tr>
<td>PtdInsP₂</td>
<td>0.0271 ± 0.136*</td>
<td>0.7190 ± 0.0147</td>
<td>0.6043 ± 0.1126</td>
<td>0.5936 ± 0.0980*</td>
<td></td>
</tr>
<tr>
<td>Efflux</td>
<td>41.63 ± 5.89*</td>
<td>167.69 ± 41.46</td>
<td>201.84 ± 9.81*</td>
<td>236.34 ± 24.86*</td>
<td></td>
</tr>
</tbody>
</table>

The conditions examined. There was a modest (1.8-fold) increase in the incorporation of label into free myo-inositol in EGF-treated cells compared with control cells (P < 0.025). Otherwise there were no changes in the immediate destinations of myo-inositol following EGF treatment when compared with controls. TSH treatment, however, resulted in a large increase in the incorporation of label into free myo-inositol (8.0-fold rise; P < 0.005), increases in incorporation into GroPIns, InsP and InsP₂, and a 4.1-fold increase in the incorporation of myo-inositol into PtdIns (P < 0.01); the levels of the remaining lipids were unchanged. The increased incorporation of label into InsP and InsP₂ again suggests the presence of a PLC-mediated signalling event. The increased incorporation of label into PtdIns suggests that, in TSH-treated cells, the rate of PtdIns synthesis is increased over control cells.

Simultaneous treatment with TSH and EGF resulted in an increase in the incorporation of label into free myo-inositol (4.6-fold, P < 0.01), an increase in incorporation into GroPIns, and an increase in the incorporation of myo-inositol into PtdIns (3.1-fold rise, P < 0.01). The increase in incorporation of label into GroPIns in TSH plus EGF-treated cells was more than additive.

The results obtained with pulse labelling of TSH-treated cells suggested that there was an increased synthesis of PtdIns, yet the equilibrium labelling study showed no alteration in PtdIns levels. One possible explanation of these results is that PtdIns is rapidly turning over in TSH-treated cells. To examine this, pulse–chase labelling studies were performed.

Effects of TSH, EGF and TSH plus EGF on myo-inositol metabolism: pulse–chase labelling study

Thyrocytes were pulse-labelled with [³H]myo-inositol for 15 min, followed by a chase with 50 µM unlabelled myo-inositol for 15 min. Data are shown in Table 3. After the 15 min chase the amount of label found in the free myo-inositol pool had significantly decreased from that found after the 15 min pulse, suggesting that myo-inositol entering this pool is quickly diverted elsewhere. The rate of flux of myo-inositol through this pool was similar in insulin- and EGF-treated cells, but was greatly increased in TSH- and TSH plus EGF-treated cells. Label from the myo-inositol pool appeared in GroPIns, InsP, InsP₂, PtdIns, PtdInsP, and PtdInsP₂, but the majority of it (91–96 % in all cases) reappeared in the extracellular medium (Table 3), suggesting that there was rapid cycling of myo-inositol between the intracellular and extracellular media. A similar finding was observed with GroPIns. This pool appeared to be rapidly turning over and there was a greater turnover rate in TSH- or TSH plus EGF-treated cells than there was for insulin- or EGF-treated cells. The incorporation of label into InsP and InsP₂ respectively increased slightly after the 15 min chase or remained the same. Although levels of InsP and InsP₂ in TSH-treated cells were increased, their rates of turnover were the same as in the control cells.

In all cases there was a significant increase in label in PtdIns after the 15 min chase compared with the 15 min pulse, indicating that a proportion of the myo-inositol was used for PtdIns synthesis. The rate of synthesis of PtdIns was higher in TSH- or TSH plus EGF-treated cells than in insulin- or EGF-treated cells. Increased amounts of label were found in PtdInsP and unchanged amounts in PtdInsP₂ in all cases, indicating the synthesis of these lipids from PtdIns. In TSH- or TSH plus EGF-treated cells the increase in label found in PtdIns at 30 min was half that found in EGF- or insulin-treated cells (1.7-fold increase versus 3.4-fold increase), suggesting that in these cells a proportion of the PtdIns is metabolized rapidly and hence at 30 min the label has been diverted elsewhere.

Arachidonic acid metabolism

The increased turnover of GroPIns (Tables 2 and 3) is consistent with PLA₂-mediated turnover of PtdIns. Therefore metabolism of arachidonic acid, which is a product of this reaction, was examined. Thyrocytes were equilibrium labelled with [¹⁴C]acetate and were treated with TSH (0.3 nM), EGF (1 × 10⁻⁸ M), or TSH plus EGF (0.3 nM plus 1 × 10⁻⁸ M) in the presence of insulin (1 µg/ml) for 24 h and eicosanoids were extracted. Arachidonic
acid was the only major eicosanoid detected; the only other spots on the TLC plate corresponded to cholesterol esters, triacylglycerols, and phospholipids. There was no change in the amount of $^{14}$C-labelled arachidonic acid under any of the conditions described (Table 4). To eliminate the possibility that arachidonic acid was being rapidly metabolized, indomethacin (15 μM), a cyclo-oxygenase inhibitor, was added for the final 2 h of culture. This also had no effect on the levels of arachidonic acid.

To determine whether turnover of arachidonic acid could explain these data, thyrocytes were treated with TSH (0.3 nM), EGF (1 x $10^{-8}$ M) or TSH plus EGF in the presence of insulin (1 μg/ml) for 24 h. [1-$^{14}$C]Acetic acid, sodium salt (2.5 μCi/10$^6$ cells), and indomethacin (15 μM) were added for the final 2 h of culture and eicosanoids were extracted. The results (Table 4) show that in the presence of TSH or TSH plus EGF there is increased turnover of (but not net production of) arachidonic acid. These results are in accord with the increased turnover of PtdIns and GroPIns under these two conditions. The data suggest that the TSH-mediated increase in myo-inositol transport $V_{max}$ is associated with the PLAr-mediated turnover of PtdIns and an increased turnover of arachidonic acid.

### Discussion

Previous studies have suggested that the rate of myo-inositol transport regulates the intracellular concentration of myo-inositol [24,25] and that the rate of PtdIns synthesis is dependent on the extracellular myo-inositol concentration [45–48]. The data presented in this study confirm those observations and further demonstrate that the rate of PtdIns synthesis is ultimately regulated by the rate of myo-inositol transport. The increase in GroPIns turnover associated with the increased PtdIns turnover demonstrates that PtdIns turnover occurs via hydrolysis and resynthesis of this lipid and not via the PtdIns–inositol exchange reaction. The data suggest the presence of specific pools of myo-inositol which are preferentially used for PtdIns synthesis. Similar observations have been made in nerve and renal epithelium [47,48]. The enzyme PtdIns synthase (CDP-diacylglycerol-myoinositol-3-phosphatidyltransferase; EC 2.7.8.11; [49]) regulates PtdIns synthesis. This enzyme is found in the endoplasmic reticulum [50], and a PtdIns synthase has been described in the plasma membrane of a pituitary tumor cell line [51]. The plasma membrane enzyme has a considerably lower $K_m$ for myo-inositol (67 μM) than the endoplasmic reticulum enzyme (1.5–4.5 mM; [50]) which would allow it to regulate the turnover of plasma membrane PtdIns in response to small (μM) changes in the local myo-inositol concentration.

These observations provide an explanation for early results using dog and sheep thyroid slices in which increased PtdIns turnover was noted following TSH stimulation [20]. They also link with previous observations that TSH can cause increases in the non-equilibrium levels of arachidonic acid [9,52–54]. GroPIns is a product of the metabolism of PtdIns by PLA$_2$ followed by a lysophospholipase [44]. PtdIns is enriched for arachidonic acid in the 2-position [55]; therefore the production of arachidonic acid and GroPIns associated with PtdIns turnover strongly suggests that TSH induced the PLAr-mediated turnover of PtdIns. The effects are also found during the differentiation of HL60 cells towards monocytes, where there is an increase in the rate of myo-inositol transport [25] and in the levels of arachidonic acid [56].

An increase in the equilibrium levels of GroPIns has previously been postulated as either a marker for the transformation of cells with an oncogene [44] or as a product of the differentiation of certain cell types [43]. Elevation of the levels of GroPIns has been observed during HL60 cell differentiation towards neutrophils, and the differentiation of normal myeloid blast cells towards monocytes [26,42,43]. In the present study an increase in the equilibrium levels of GroPIns was not observed in TSH-stimulated (differentiating) cells, or in EGF-stimulated (growing) cells, but was observed in the presence of the two antagonistic stimuli. Elevations in the level of GroPIns may therefore be a marker of cross-talk between several cellular signalling pathways.

The data demonstrate that in sheep and human thyrocyte cultures the rate of myo-inositol transport can be regulated during growth and differentiation. This phenomenon has previously been observed in myeloid cells differentiating towards monocytes or neutrophils [24,25], and in neuroblastoma cells [57]. However, this is the first report of regulation of myo-inositol transport during the growth and differentiation of non-transformed cells in response to physiological ligands. Regulation of the availability of myo-inositol may be a generalized feature of growth and differentiation. In human cells the up-regulation of myo-inositol transport was achieved via increased steady-state levels of the mRNA for the transporter. Together with the increased $V_{max}$ and the time course for the increase, these data suggest that up-regulation occurred via an increase in the number of transporters at the cell surface. Previous studies have also suggested that myo-inositol transport is regulated by altering the number of transporters at the cell surface [34,58].

The differences in the fate of the transported myo-inositol following TSH or EGF treatment illustrate the profound pheno-
typic differences that these ligands induce. In the EGF-treated cells there are few significant changes in the metabolites of myo-inositol compared with controls, and the increased uptake is probably related to the maintenance of the intracellular concentration of myo-inositol as the cells enlarge in preparation for division. Following TSH treatment, the increased cycling of myo-inositol and PL_{A2}-mediated turnover of PtdIns suggest activation of further signalling pathways involved in cell differentiation. Generally, dbcAMP produced the same or similar results as TSH stimulation, suggesting that the TSH effects were mediated through cyclic AMP production. The peak increase in myo-inositol transport occurred 24 h after TSH, and the same is also true for the turnover of PtdIns and arachidonic acid. Membrane-associated signalling events usually occur within seconds of the original stimulus and are attenuated, at most, several hours afterwards [41]. The protracted time course of the TSH-induced PtdIns turnover may be required for the temporal regulation of events many hours after the initiating stimulus. A recent report on FRTL5 cells shows that TSH induces an increase in diacylglycerol content and PKC activity 24 h post-addition [59]. Results described in this study demonstrate that there is more to the involvement of myo-inositol in cell signalling than the PLC-mediated turnover of PtdIns(4,5)P_{2}, and that signalling events may be closely regulated hours and days after the generation of the original second messenger.

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REFERENCES


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myo-inositol transport in growth and differentiation