Phosphatidylinositol 3-kinase, protein kinase B and ribosomal S6 kinases in the stimulation of thyroid epithelial cell proliferation by cAMP and growth factors in the presence of insulin

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The proliferation of most normal cells depends on the co-operation of several growth factors and hormones, each with a specific role, but the key events involved in the action of each necessary stimulant remain largely uncharacterized. In the present study, the pathways involved in the mechanism(s) of co-operation have been investigated in primary cultures of dog thyroid epithelial cells. In this physiologically relevant system, thyroid stimulating hormone (TSH) acting through cAMP, epidermal growth factor (EGF) and phorbol esters (such as PMA) induce DNA synthesis. Their effect requires stimulation of the insulin-like growth factor-1 (IGF-1) receptor by either IGF-1 or insulin, which are not themselves mitogenic agents. In contrast, hepatocyte growth factor (HGF) is itself fully mitogenic. The results of the study demonstrate that cAMP, EGF, HGF and PMA stimulate p70 ribosomal S6 kinase (p70 S6 kinase). However, insulin/IGF-1 also stimulate p70 S6 kinase. Thus stimulation of p70 S6 kinase might be necessary, but is certainly

not sufficient, for the induction of DNA synthesis and is not specific for any stimulated pathway. In contrast, phosphatidylinositol 3-kinase (PI 3-kinase) and protein kinase B (PKB) activation by insulin and HGF is strong and sustained, whereas it is weak and transient with EGF and absent in the presence of TSH or PMA. These findings suggest that: (i) stimulation of PI 3-kinases and/or PKB is not involved in the cAMP-dependent pathways leading to thyrocyte proliferation, or in the action of PMA, (ii) the stimulation of the PI 3-kinase/PKB pathway may account for the permissive action of insulin/IGF-1 in the proliferation of these cells, and (iii) the stimulation of this pathway by HGF may explain why this agent does not require insulin or IGF-1 for its mitogenic action.

Key words: DNA synthesis, hepatocyte growth factor, rapamycin, thyrotropin, wortmannin.

INTRODUCTION

The regulation of cell proliferation has been much studied in cell lines. Such models are only distantly related to normal cells. Moreover, the roles of the various pathways studied have been found to be different, or even the converse, in one cell type and another. In the study of physiological control it is, therefore, essential to establish the regulatory network of each normal cell type. Some form of co-operation is generally needed between different growth factors in order to achieve the stimulation of division of normal cells. In particular, the almost ubiquitous activity of insulin-like growth factor (IGF-1) in supporting cell proliferation triggered by growth factors is illustrated by the almost universal requirement of IGF-1 or high concentrations of insulin [1] as a supplement in serum-free culture media for most normal cell types [1–3]. The positive role of cAMP in cell cycle progression provides interesting examples of such a co-operation in many different epithelial cells [4]. The mechanism(s) of this co-operation is unclear, mainly because the key events involved in each signalling cascade have not been delineated. In this study, we have investigated the role of phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase B (PKB) and p70 ribosomal protein S6 kinase (p70 S6 kinase) in this regard. Activation of p70 S6 kinase is involved in the regulation of both protein and DNA synthesis. p70 S6 kinase was identified on the basis of its ability

to phosphorylate the 40 S ribosomal protein S6 *in vitro* [5]. A number of other kinases, including the growth-regulated 90 kDa ribosomal S6 kinase (p90 S6 kinase), which is phosphorylated and activated by the p42/p44 mitogen-activated protein kinases (MAPKs), can also phosphorylate S6 in *in vitro* systems, but it has been shown that, in the cell, this function is mostly performed by p70 S6 kinase [5,6]. S6 phosphorylation is thought to increase the rate of synthesis of certain proteins which are required for efficient G₁ progression and whose mRNAs contain a polypyrimidine tract at the 5' end [5]. p70 S6 kinase has been suggested to play an essential role throughout G₁, as its inhibition by microinjection of neutralizing antibodies [5] or by the immunosuppressant rapamycin severely compromised the ability of various types of cells to progress through the G₁ phase of the cell cycle [7]. Complexed to FK506-binding-protein ('FKBP'), rapamycin interacts with the mammalian target of rapamycin (mTOR) and blocks the activation of p70 S6 kinase in response to all stimuli so far studied [7,8]. p70 S6 kinase activation in response to stimuli requires a hierarchical phosphorylation at multiple serine/threonine sites [8,9]. A role for PI 3-kinase and protein kinase B (PKB) in this activation has been suggested but remains controversial and probably depends on the nature of the agonist and of the cell type [8,10–12]. PKB itself is a direct target of PI 3-kinase although it can also be activated by PI 3-kinase independent pathways [13,14].

Abbreviations used: IGF-1, insulin-like growth factor 1; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; p70 S6 kinase, p70 ribosomal S6 kinase; p90 S6 kinase, p90 ribosomal S6 kinase; MAPK, mitogen-activated protein kinase; TSH, thyroid stimulating hormone; PKA, protein kinase A; EGF, epidermal growth factor; HGF, hepatocyte growth factor; mTOR, mammalian target of rapamycin.

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Dog thyroid follicular cells (thyrocytes) in primary culture are representative of their *in vivo* counterpart [15]. In these cells, cAMP, as a second messenger for thyrotropin (thyroid stimulating hormone; TSH), positively controls proliferation and differentiation through pathways that require protein kinase A (PKA) activity and other mechanisms [15,16]. DNA synthesis can also be induced through cAMP-independent mechanisms, in response to epidermal growth factor (EGF), hepatocyte growth factor (HGF) and phorbol esters, coinciding with an inhibition of differentiation [15]. The mitogenic effects of TSH, EGF and PMA, but not those of HGF, require IGF-1 receptor stimulation by IGF-1 or high concentrations of insulin [17,18]. In themselves, IGF-1 and insulin have little mitogenic action. As the real physiological stimulator of thyroid cells is TSH, the role of insulin/IGF-1 is described as permissive for TSH and thus, by extension, for EGF and PMA action. Until now the mechanism of the permissive effect of insulin/IGF-1 has been little studied.

Whereas signalling pathways stimulated by growth factors acting on receptor tyrosine kinases or on protein kinase C have been relatively well delineated, little is known about those stimulated by cAMP. In an earlier study we have shown that stimulation of dog thyrocytes by TSH did not result in the activation of any member of the MAPK family ([19,20]; F. Vandeput, unpublished work), suggesting a distinct signalling pathway. Recent studies using cell lines demonstrated stimulation of PKB by cAMP [13,21].

In the present work, in order to unravel the mechanism(s) of co-operation leading to cell proliferation, we have studied the effect of TSH acting through cAMP and of growth factors acting through receptor tyrosine kinases (insulin/IGF-1, EGF, HGF) or through protein kinase C (PMA) on the activities of p70 and p90 S6 kinases, PKB and PI 3-kinase in primary cultures of dog thyroid cells.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, MCBBD104 medium, penicillin, streptomycin and amphotericin B (Fungizone) were obtained from GIBCO (Paisley, Scotland, U.K.). Bovine insulin, PMA, murine EGF and bovine TSH were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Forskolin was from Calbiochem (La Jolla, CA, U.S.A.). Human recombinant HGF (hrHGF) was a gift from T. Nakamura and K. Matsuto (Osaka University Medical School, Osaka, Japan). Rapamycin and wortmannin were gifts from Wyeth-Ayerst Research (Princeton, CN, U.S.A.) and Sandoz Pharma Ltd. (Basel, Switzerland) respectively. Wortmannin was also purchased from Sigma, as well as LY294002 and PtdIns. The p70 S6 kinase antibodies used were raised against the N-terminal (amino acids 1–31) [22] or the C-terminal peptide sequence specific for p70 S6 kinase (amino acids 485–502) (C-18; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) of rat p70 S6 kinase. Polyclonal antibodies to p90 S6 kinase (C-21), pRb (C-15), p107 (C-18) and p130 (C-20) were purchased from Santa Cruz Biotechnology; polyclonal antibodies to PKB were from Upstate Biotechnology (Lake Placid, NY, U.S.A.), and anti-phosphotyrosine monoclonal antibody (PY20) from Transduction Laboratories (Lexington, KY, U.S.A.). The S6 substrate and PKA inhibitor peptides were supplied by Santa Cruz Biotechnology, the crosstide peptide by Upstate Biotechnology. Secondary antibodies, enhanced chemiluminescence reagents, [³²P]P, [^γ-³²P]ATP and [³H]thymidine were from Amersham International (Little Chalfont, Bucks., U.K.). Protein A-Sepharose CL4B and Protein G were purchased from Pharmacia,

and phosphocellulose (Whatman P-81) was from Whatman (Maidstone, Kent, U.K.). Silica gel 60 aluminium TLC plates were obtained from Merck.

Primary culture

Cells were obtained from dog thyroid tissue as detailed previously [15]. Thyrocytes were cultured in monolayer (2×10^4 cells/cm²) in the following medium: Dulbecco's modified Eagle's medium/Ham's F12 medium/MCBD 104 medium (2:1:1, by vol.) supplemented with 40 µg/ml ascorbic acid, antibiotics, and with or without insulin (5 µg/ml). After culture for 4 days, the cells were stimulated for various periods of time by different agents. In some cases, cells were treated with rapamycin (2 mM stock in ethanol), wortmannin (10 µM stock in DMSO) or LY294002 (5 M stock in DMSO) for 30 min before activation.

DNA synthesis

After 4 days of culture in the absence or presence of insulin (5 µg/ml), dog thyroid cells were incubated for 48 h with the mitogenic agents and with [³H]thymidine (10 µCi/ml, 3×10^{-5} M) and deoxycytidine (10^{-4} M) for the final 24 h. The number of cells entering into DNA synthesis was estimated by the frequency of [³H]thymidine-labelled nuclei, as revealed by autoradiography. In each duplicate dish, at least 1000 nuclei chosen at random in different fields were counted blind [15].

S6 kinases assay

p70 S6 kinase and p90 S6 kinase activities were measured as described previously [22], except that 100 µM RRRLLSLRA peptide was used as substrate [23], and the reaction was terminated by the addition of acetic acid to a final concentration of 30%. The amount of ³²P-label incorporated into the peptide substrate was determined by scintillation spectroscopy [24]. Controls were processed similarly in the absence of protein kinase. Protein determinations were performed as described previously [25] with BSA as standard.

PKB assay

After stimulation, the cells were solubilized in a buffer composed of 120 mM NaCl, 25 mM Tris/HCl (pH 7.6), 50 mM NaF, 1 mM benzamidine, 1 mM EDTA, 15 mM sodium pyrophosphate, 1 mM EGTA, 20 mM 2-glycerophosphate, 2 mM Na₃VO₄, 0.5% sodium deoxycholate, 1% (v/v) Nonidet P-40, 60 µg/ml Pefabloc, 100 mM okadaic acid and 10 µg/ml each of leupeptin and pepstatin. Samples containing 200 µg protein were subjected to immunoprecipitation using a polyclonal antibody against PKB coupled to Protein G-Sepharose. After washing the immunoprecipitates four times with 50 mM Tris/HCl (pH 7.5), 50 mM NaF, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM EGTA, 10 mM 2-glycerophosphate, 1 mM Na₃VO₄, 0.1% (v/v) Triton X-100, and twice with kinase buffer [20 mM Mops (pH 7.2), 5 mM EGTA, 25 mM 2-glycerophosphate, 1 mM dithiothreitol, 1 mM Na₃VO₄], kinase activity was assayed in a final volume of 30 µl kinase buffer containing 50 µM ATP, 75 mM MgCl₂, 30 µM crosstide peptide, 17 µM protein kinase A inhibitor and 3 µCi [^γ-³²P]ATP. The phosphorylation reaction was allowed to proceed for 15 min at 30 °C. The amount of ³²P incorporated into the peptide substrate was determined by scintillation spectroscopy as described previously.

PI 3-kinase assay

PI 3-kinase activity was measured on anti-phosphotyrosine immune complexes essentially as described previously [26]. The

reactions were initiated by the addition of 50 μ M ATP, 5 mM $MgCl_2$ and 2 μ Ci [γ - ^{32}P]ATP (final concentrations) and stopped after 20 min at 30 °C with 15 μ l of 4 M HCl and 250 μ l of chloroform/methanol (1:1, v/v). The lower organic phase was washed with 150 μ l of methanol/1 M HCl (1:1, v/v) and dried. Phospholipids were resuspended in 10 μ l of chloroform, separated by TLC on aluminium-backed silica gel 60 plates pretreated with 1% potassium oxalate in the solvent system chloroform/methanol/acetone/acetic acid/water (60:20:23:18:11, by vol.) and detected by autoradiography.

Gel electrophoresis and immunodetection of proteins

At the end of the culture period, total cellular proteins were separated according to molecular mass by SDS/PAGE (6.5% gel) and immunodetected after Western blotting as described previously [25]. Immunoblots were developed using ^{125}I -labelled Protein A or the ECL[®] kit (Amersham) and subjected to autoradiography.

Analysis of intracellular levels of 3-phosphorylated phosphoinositides

Production of 3-phosphorylated lipids *in vivo* was determined essentially as described previously [27,28]. Briefly, thyroid cells were prelabelled with [^{32}P]P_i (0.3 mCi/ml) for 3 h and then stimulated for various periods of time with the appropriate agents. Cells were scraped into 1 M HCl and phospholipids were extracted into HCl/methanol/chloroform (47:48:3, by vol.) in the presence of 25 mM EDTA, butylated hydroxytoluene antioxidant and 30 μ g of a mixture of brain phosphoinositides. The phospholipids were then converted to water-soluble deacylated derivatives [28], separated on a Partisphere SAX (Whatman) HPLC column and the radioactivity was estimated [29].

RESULTS

Rapamycin and wortmannin inhibit DNA synthesis

To investigate the role of mTOR/p70 S6 kinase and PI 3-kinase activation in the pathways leading to thyroid cell proliferation, the effect of inhibitors of these enzymes on DNA synthesis has been studied. Table 1 shows that rapamycin and wortmannin substantially reduced DNA synthesis in cells stimulated by TSH,

Table 1 Inhibition of DNA synthesis in thyrocytes by rapamycin and wortmannin

After 4 days of culture in medium supplemented with or without (*) 5 μ g/ml insulin, the cells were untreated or preincubated with 20 nM rapamycin or 100 nM wortmannin for 30 min before the addition of the various reagents for 48 h. During this time, because of the transient effect of wortmannin, 100 nM was added every 12 h up to 400 nM [48,49]. [3H]Thymidine was present for the last 24 h. Results are means $\pm$ S.D. of at least three different experiments performed in duplicate with similar results.

Reagent	Labelled nuclei (%)		
	No pretreatment	Rapamycin	Wortmannin
Control	11.3 $\pm$ 4.4	0.8 $\pm$ 0.5	0.3 $\pm$ 0.3
TSH (1 m-unit/ml)	52.4 $\pm$ 15.9	23.4 $\pm$ 9.5	13.1 $\pm$ 5.2
Forskolin (10 ⁻⁵ M)	63.4 $\pm$ 7.5	25.6 $\pm$ 6.7	11.9 $\pm$ 3.8
EGF (25 ng/ml)	51.2 $\pm$ 2.5	12.7 $\pm$ 2.0	28.0 $\pm$ 10.3
PMA (10 ng/ml)	41.2 $\pm$ 6.1	3.2 $\pm$ 3.1	7.3 $\pm$ 3.1
HGF (50 ng/ml)	69.9 $\pm$ 3.4	21.9 $\pm$ 2.5	23.4 $\pm$ 1.4
*Control	1.2 $\pm$ 0.6	—	0.2 $\pm$ 0.3
*HGF (50 ng/ml)	33.5 $\pm$ 4.5	—	1.4 $\pm$ 0.5

Figure 1 Inhibition of pRb, p107 and p130 phosphorylation by rapamycin and wortmannin

Anti-pRb, -p107, -p130 immunoblots of whole cell extracts. Dog thyrocytes were cultured for 4 days in basal medium supplemented with 5 μ g/ml insulin. They were then pretreated with 20 nM rapamycin or 100 nM wortmannin for 30 min prior to stimulation by EGF (25 ng/ml), PMA (TPA; 10 ng/ml) or TSH (1 m-unit/ml) for 24 h or remained in control condition (C). A second addition of wortmannin was made 12 h later. A 40 μ g sample of total cellular proteins was loaded per lane. Arrows indicate the position of the hypophosphorylated form of pRb and p107, and of the faster migrating hypophosphorylated form of p130. In the same representative experiment, the proportion (%) of [3H]thymidine labelled nuclei was determined in duplicate with similar results for each experimental condition (N*).

Table 2 Inhibition of DNA synthesis in dog thyrocytes by later addition of rapamycin and wortmannin

After 4 days of culture in medium supplemented with 5 μ g/ml insulin, thyrocytes were stimulated by various reagents for 48 h with or without 20 nM rapamycin or 400 nM wortmannin, which were added 12 h later. [3H]Thymidine was present for the final 24 h. The results are the means $\pm$ S.D. of at least three different experiments performed in duplicate with similar results.

	Labelled nuclei (%)		
	No pretreatment	Rapamycin	Wortmannin
Control	9.9 $\pm$ 2.5	0.8 $\pm$ 0.4	0.9 $\pm$ 0.7
EGF (25 ng/ml)	36.2 $\pm$ 1.0	11.7 $\pm$ 3.3	12.8 $\pm$ 0.4
PMA (10 ng/ml)	28.7 $\pm$ 3.2	4.8 $\pm$ 2.5	6.1 $\pm$ 1.8
TSH (1 m-unit/ml)	47.9 $\pm$ 1.1	14.4 $\pm$ 4.1	18.4 $\pm$ 0.4

forskolin, EGF, HGF or PMA in the presence of insulin, as in control cells. Figure 1 represents an experiment in which both rapamycin and wortmannin strongly decreased mitogen-induced proliferation of dog thyrocytes, coincident with the prevention of the hyperphosphorylation of pRb, p107 and p130, as demonstrated by the lack of appearance of the slower migrating form(s) of the proteins. This finding is in agreement with our previous study where we showed that the extent of hyperphosphorylation of these proteins correlated well with the actual induction of DNA synthesis by the mitogenic agents tested [25]. This inhibition is not a consequence of an up-regulation of the cyclin dependent kinase (cdk)-inhibitor p27^{kip1} as no change in its level of expression was observed after treatment of the cells with wortmannin or rapamycin (not shown). Table 1 also shows that wortmannin provoked a strong inhibition of the DNA synthesis induced by HGF in the absence of insulin in the culture medium. Table 2 shows that the inhibition by rapamycin or wortmannin of DNA synthesis was still present when these inhibitors were added 12 h after the stimulatory agents, i.e. during the second part of the prereplicative phase (DNA synthesis starts after 16 to 18 h) [30].

Figure 2 Increase of p70 S6 kinase electrophoretic mobility by rapamycin and wortmannin

Anti-p70 S6 kinase immunoblots of whole cell extracts. After 4 days of culture in the absence of insulin, the thyrocytes were pretreated or not with 20 nM rapamycin or 100 nM wortmannin for 30 min prior to exposure to EGF (25 ng/ml), PMA (TPA; 10 ng/ml), TSH (1 m-unit/ml), forskolin (Fo) (10^{-5} M), HGF (50 ng/ml) or insulin (5 μ g/ml) for 30 min or remained in control condition (C). A 50 μ g sample of total cellular proteins was loaded per lane. The arrow indicate the position of the hypophosphorylated form of p70 S6 kinase.

Table 3 Incorporation of [3 H]thymidine into the nuclei of thyrocytes cultured in the absence of insulin

After 4 days of culture in medium lacking insulin, thyrocytes were preincubated with 100 nM wortmannin or 15 μ M LY294002 for 30 min before the incubation with various agents for 48 h. During this time, because of the transient effect of wortmannin, 100 nM was added every 12 h, up to 400 nM. [3 H]Thymidine was present for the final 24 h. The results are the means $\pm$ S.D. of data from at least three independent experiments performed in duplicate with similar results.

Reagent	Labelled nuclei (%)
Control	3.6 $\pm$ 3.1
Insulin (5 μ g/ml)	13.3 $\pm$ 4.1
TSH (1 m-unit/ml)	13.9 $\pm$ 5.3
Forskolin (10^{-5} M)	8.7 $\pm$ 1.8
PMA (10 ng/ml)	3.9 $\pm$ 3.6
EGF (25 ng/ml)	5.7 $\pm$ 4.5
HGF (50 ng/ml)	32.9 $\pm$ 11.2
TSH + insulin	45.0 $\pm$ 9.5

Table 4 Effect of various reagents, and influence of rapamycin, wortmannin and LY294002 on p70 S6 kinase activity

After 4 days of culture in the absence of insulin, dog thyrocytes were preincubated with 20 nM rapamycin, 100 nM wortmannin or 15 μ M LY294002 for 30 min before incubation with various reagents for 30 min. The results of p70 S6 kinase assays are presented as fold stimulation above control and are means $\pm$ S.E.M. of three independent experiments. p70 S6 kinase activity in control cells was 4.41 $\pm$ 0.70 units/mg of protein when an S6 peptide was used as substrate.

Reagent	p70 S6 kinase activity			
	No pretreatment	Rapamycin	Wortmannin	LY294002
Control	1.00	0.01 $\pm$ 0.01	0.11 $\pm$ 0.06	0.16 $\pm$ 0.09
Insulin (5 μ g/ml)	3.62 $\pm$ 0.17	—	0.25 $\pm$ 0.07	1.52 $\pm$ 0.19
TSH (1 m-unit/ml)	2.29 $\pm$ 0.14	0.05 $\pm$ 0.06	0.67 $\pm$ 0.05	0.19 $\pm$ 0.03
Insulin + TSH	3.54 $\pm$ 0.22	—	0.63 $\pm$ 0.29	1.01 $\pm$ 0.21
Forskolin (10^{-5} M)	2.28 $\pm$ 0.22	—	1.03 $\pm$ 0.24	0.30 $\pm$ 0.05
EGF (25 ng/ml)	2.65 $\pm$ 0.15	0.14 $\pm$ 0.01	0.30 $\pm$ 0.09	0.60 $\pm$ 0.16
PMA (10 ng/ml)	2.58 $\pm$ 0.24	0.01 $\pm$ 0.01	1.06 $\pm$ 0.09	0.92 $\pm$ 0.22
HGF (50 ng/ml)	3.37 $\pm$ 0.30	—	—	—

Effect of different agents on p70 and p90 S6 kinase activities

Dog thyrocytes in culture require a high insulin concentration for most mitogens to induce DNA synthesis [15]. The effect of these agents acting alone on p70 S6 kinase activation was assessed from p70 S6 kinase immunoblots of total cell lysates, by the relative mobility of this protein following SDS/PAGE (Figure 2), reflecting different degrees of p70 S6 kinase phosphorylation [9]. Before stimulation, thyrocytes were cultured for 4 days in the absence of insulin. In these culture conditions, the agents have little to no mitogenic effect, except HGF whose action on proliferation does not require the presence of insulin (Table 3). Figure 2 shows that in control cells four forms of p70 S6 kinase can already be distinguished, the faster migrating one being the most abundant. Both cAMP-independent agents (EGF, HGF, insulin and PMA) and cAMP-dependent agents provoked a retardation in the migration of p70 S6 kinase, the slowest migrating band corresponding to the catalytically active enzyme [9]. The presence of a small amount of fully phosphorylated p70 S6 kinase in control cells could result from a small activation of this enzyme following attachment and spreading of thyroid cells on Petri dishes [31]. Inhibition of p70 S6 kinase phosphorylation by rapamycin or wortmannin was reflected by the decreased intensity of the slower electrophoretic mobility band of the kinase and by the corresponding increase of intensity of the faster migrating band (Figure 2).

In order to assess the potency of the different agents studied on p70 or p90 S6 kinase activity, *in vitro* kinase assays were performed using immunoprecipitates of these enzymes from

control or stimulated cells. Table 4 shows that (i) insulin and HGF are the most potent activators of p70 S6 kinase activity in these cells (3.62 and 3.37 fold respectively); (ii) TSH, EGF and PMA stimulate p70 S6 kinase activity to about the same extent (2.29, 2.65 and 2.58 fold respectively); (iii) stimulation by TSH of p70 S6 kinase activity is reproduced by forskolin, a general activator of adenylate cyclase; (iv) simultaneous addition of TSH and insulin resulted in a comparable activation to that obtained with insulin alone (3.54 fold). This is in contrast with the results of Withers et al. [32] who observed, in Swiss 3T3 cells, that the mitogenic combination of forskolin and insulin induced an additive stimulation of p70 S6 kinase activity. Time course experiments revealed that TSH or EGF induced a biphasic activation of p70 S6 kinase, peaking after about 30 and 120 min (not shown). The enzyme activity was already increased after 5 min and remained over control for at least 8 h (not shown). On the other hand, in accordance with our results on MAPK phosphorylation [18,20,33] all these agents except TSH and forskolin stimulate p90 S6 kinase activity, EGF, HGF and PMA having about the same potency, insulin being the weakest stimulator (Table 5). Moreover, TSH did not affect the stimulation, by insulin, of the p90 S6 kinase activity (Table 5). These results thus confirm that in dog thyroid cells, in contrast to many other cell types [34,35], cAMP had no inhibitory or stimulatory effect on the MAPK/p90 S6 kinase pathway. Rapamycin (20nM) lowered the basal level of p70 S6 kinase activity in control cells and almost totally abolished p70 S6 kinase activation by TSH,

Table 5 Effect of various reagents on p90 S6 kinase activity

After 4 days of culture in the absence of insulin, thyrocytes were stimulated with various reagents for 20 min. The results of p90 S6 kinase assays are presented as fold stimulation above control and are means $\pm$ S.E.M. of three independent experiments. p90 S6 kinase activity in control cells was 7.82 ± 0.92 units/mg of protein when an S6 peptide was used as substrate.

Reagent	p90 S6 kinase activity
Control	1.00
TSH (1 m-unit/ml)	0.88 ± 0.05
Forskolin (10^{-5} M)	0.82 ± 0.12
EGF (25 ng/ml)	2.99 ± 0.76
HGF (50 ng/ml)	2.54 ± 0.50
PMA (10 ng/ml)	2.36 ± 0.51
Insulin (5 μ g/ml)	2.06 ± 0.12
Insulin + TSH	2.16 ± 0.12

EGF or PMA (Table 4). The effect of wortmannin and of LY294002, was also investigated on p70 S6 kinase activity. Although treatment of the cells with LY294002 (15 μ M) resulted, as with wortmannin (100 nM), in the inhibition of p70 S6 kinase activity in control cells as well as in cells stimulated by the different agents, LY294002 seemed to be a more potent inhibitor than wortmannin of the cAMP dependent activation of p70 S6 kinase and a lesser one of the insulin activation of this kinase (Table 4). These observations have still no explanation. In any case these inhibitions suggest the implication of PI 3-kinase upstream of p70 S6 kinase. It is worth noting that there are some discrepancies between our data and those obtained by Cass and MeinKoth [36] and Cass et al. [37] in the WRT cell line as, in these cells, inhibition of the cAMP dependent p70 S6 kinase was obtained with 15 μ M LY294002 but not with 200 nM wortmannin.

Agents acting through receptor tyrosine kinases, but not TSH or PMA, stimulate PKB activity

As it is generally accepted that PKB is a primary downstream target of the different isoforms of PI 3-kinase [10,38], we investigated the activity of this kinase in dog thyrocytes. The cells were cultivated 4 days without insulin before the addition of the different stimulating agents. PKB activity was measured in immune-complex assays using the crosstide peptide as substrate. Figure 3(A) showed that after 8 min of stimulation, PKB activity was increased by insulin, HGF and EGF. Insulin and HGF were the most potent stimulating agents (respectively 7.5 and 6.9 fold), followed by EGF (3.5 fold), while PMA and forskolin were without effect. The very small stimulation of PKB activity observed in the presence of TSH (1.2 fold) did not depend upon the intracellular increase in cAMP as forskolin, a general activator of adenylate cyclase, had no effect on the activity of this enzyme. Treatment of thyrocytes with wortmannin (100 nM), added to the culture medium prior to stimulation of the cells by insulin, EGF or HGF, inhibited the activity induced by these agents. As expected, rapamycin (20 nM) was without effect on PKB activation. Stimulation, by insulin, EGF and HGF, of PKB activity was rapid as it was already noticed at the first time point studied (2 min), but the duration of the EGF action was much more transient than that of insulin and HGF, which induced PKB activation for at least 4 h (Figure 3B). The kinetic analysis also showed that TSH or forskolin did not enhance PKB activity at any time point studied from 2 min to 4 h. There was a good

Figure 3 Agents acting through receptor tyrosine kinases but not TSH or PMA stimulate PKB activity

After 4 days of culture in the absence of insulin, dog thyroid cells were stimulated (A) for 8 min with insulin (5 μ g/ml), TSH (1 mU/ml), forskolin (10^{-5} M), EGF (25 ng/ml), HGF (50 ng/ml) or PMA (TPA; 10 ng/ml) or (B) for the indicated times with: insulin, ●; HGF, ○; EGF, ■; TSH, ▲; or forskolin, △. Where indicated in (A), the cells were pretreated with 100 nM wortmannin (filled bars) or 20 nM rapamycin (hatched bar) for 30 min. PKB activity was determined in an *in vitro* kinase assay as described in the Experimental section using crosstide as a substrate. Results are presented as fold stimulation above control, and are a representative (B) or means $\pm$ S.D. of at least three independent experiments (A).

correlation between these results and the effect of the different agents on PKB mobility observed in immunoblotting experiments (not shown).

Evaluation of PI 3-kinase activity *in vitro*

The PI 3-kinase activity was assayed by the capacity of the enzyme found in anti-phosphotyrosine immunoprecipitates to phosphorylate PtdIns (Figure 4). Its activity was significantly increased in the anti-phosphotyrosine immunoprecipitates from thyrocytes treated for 5 min with either insulin, HGF or EGF (Figure 4A). The effect of EGF on this activity was much weaker than that of insulin or HGF. All three agents were also shown to

Figure 4 Agents acting through receptor tyrosine kinases but not TSH or PMA stimulate PI 3-kinase activity

After 4 days of culture in the absence of insulin, dog thyroid cells were stimulated for 5 min with insulin (5 μ g/ml), EGF (25 ng/ml), TSH (1 m-unit/ml) or PMA (10 ng/ml) (TPA). Cells lysed using 1% (v/v) Nonidet-P40 were immunoprecipitated with an anti-phosphotyrosine antibody. (A) Precipitates were assayed for PI 3-kinase activity (see the Experimental section) and the 32 P-labelled lipid products were separated by TLC. PIP, PtdIns3P. (B) Precipitates were subjected to Western-blot analysis to determine the relative amount of p85 α .

Table 6 Abundance of PtdIns(3,4,5) P_3 in thyroid cells stimulated by various reagents

After 4 days of culture in the absence of insulin, dog thyroid cells were prelabelled with [32 P]P $_i$ (0.3 mCi/ml) for 3 h and stimulated for various periods of time with the appropriate reagents. Lipids were extracted and analysed as described in the Experimental section. The results are means of duplicate determinations in one representative experiment, and are expressed as a percentage of the total radioactivity. Similar results were obtained in one other experiment. N.D., below the limit of detection.

Reagent	Time	PtdIns(3,4,5) P_3
Control	—	0.064 $\pm$ 0.006
TSH (1 m-unit/ml)	2 min	0.032
	8 min	0.017
	30 min	0.030
	8 min	N.D.
Forskolin (10 $^{-5}$ M)	2 min	N.D.
	8 min	N.D.
Insulin (5 μ g/ml)	2 min	0.245 $\pm$ 0.004
	8 min	0.180 $\pm$ 0.014
EGF (25 ng/ml)	2 min	0.131 $\pm$ 0.018
	8 min	0.070 $\pm$ 0.009
HGF (50 ng/ml)	2 min	0.161 $\pm$ 0.019
	8 min	0.158
PMA (10 ng/ml)	2 min	0.078 $\pm$ 0.016
	8 min	0.055 $\pm$ 0.007

increase the amount of p85 α on Western blots of anti-phosphotyrosine immunoprecipitated material (Figure 4B), insulin being the most potent agent, followed by HGF, EGF being the weakest one. These results are consistent with the magnitude of the effect of these agents on PKB activity, and suggest that PI 3-kinase plays a minor role in EGF receptor-mediated signal transduction in dog thyrocytes. Figures 4(A) and 4(B) also show that we were unable to detect any PI 3-kinase activity or the presence of p85 α in anti-phosphotyrosine immunocomplexes from cells treated with TSH or PMA. This is in agreement with the absence of PKB stimulation by these agents (Figure 3).

Agents acting through receptor tyrosine kinases but not TSH or PMA stimulate the production of 3-phosphoinositides *in vivo*

To establish with certainty the lack of effect of TSH or PMA on the activity of any isoform of PI 3-kinase, intracellular levels of 3-phosphorylated phosphoinositides were analysed. This approach does not rely on any assumptions about how receptors may cause the increased formation of 3-phosphorylated phosphoinositides. Thyroid cells were prelabelled with [32 P]P $_i$ for 3 h and then stimulated with the different agents for 2 or 8 min. In the case of TSH, an additional time point (30 min) has been investigated as the increase in intracellular cAMP caused by this agent is maximal after about 30 min (results not shown). At the end of the culture, 32 P-labelled lipids were extracted, converted to water-soluble deacylated derivatives and quantified by HPLC. As generally accepted, we make the assumption that changes in the 32 P content of the 32 P-labelled phosphoinositides reflect changes in their concentrations. Table 6 shows that insulin and HGF are very good stimuli of PI 3-kinase and that EGF is a weaker one. The increases in the level of [32 P]PtdIns(3,4,5) P_3 in response to both insulin and HGF were observed after 2 min and were still present after 8 min of action of these agents, whereas the effect of EGF appeared quite transient, with PtdIns(3,4,5) P_3 levels almost returning to those of the control by 8 min. Insulin also provoked a measurable accumulation of [32 P]PtdIns(3,4) P_2 observed after 2 as well as after 8 min of action of the hormone (not shown). By contrast, PMA, TSH and forskolin did not increase the level of [32 P]PtdIns(3,4,5) P_3 . TSH and forskolin seemed to even reduce its basal formation.

DISCUSSION

The aim of our work was to delineate the steps of the cascades leading to thyroid cell proliferation. In our study we show that in dog thyrocytes, p90 S6 kinase is only stimulated by cAMP-independent agents. This pattern of stimulation of p90 S6 kinase is similar to the one observed for MAPK activation [18,20,33] which agrees with and supports, in the case of the thyroid cell, the concept that p90 S6 kinase participates in Ras/MAPK-mediated signal transduction [6]. The absence of stimulation of this pathway by the cAMP cascade shows that such a stimulation is not necessary for mitogenicity.

cAMP-induced stimulation of p70 S6 kinase activity has been previously reported by Withers et al. [32] in the Swiss 3T3 cell line and by Cass et al. [36,37] in the WRT cell line. In this work, we show by mobility shift and immune-complex kinase assays that, in primary cultures of dog thyroid cells, which represent the best studied physiological model of the cAMP pathway, p70 S6 kinase is indeed phosphorylated and activated by the cAMP-dependent agents, TSH and forskolin, and also by the cAMP-independent agents, EGF, HGF, PMA or insulin, although among them only HGF is mitogenic by itself. In dog thyroid cells, p70 S6 kinase activation thus represents the first common step of all the signalling pathways involved in proliferation: the mitogenic pathways which require insulin action (TSH, forskolin, EGF, PMA), the permissive but non-mitogenic insulin pathway, and the fully mitogenic pathway (HGF).

The first approach to identify participants in the mTOR/p70 S6 kinase pathway was to use the inhibitors rapamycin and wortmannin or LY294002 which have been reported to be specific inhibitors of mTOR [7] and of PI 3-kinase [39] respectively. In agreement with the results obtained in other cell types [7], we have found that rapamycin almost completely blocked the basal phosphorylation and activity of p70 S6 kinase as well as its stimulation by the different agents acting on dog thyroid cells. Wortmannin and LY294002 also decreased cAMP-

independent and cAMP-dependent phosphorylation and activation of p70 S6 kinase in these cells, although the cAMP-dependent p70 S6 kinase activity was only partially reduced by wortmannin in the WRT cell line [37]. The inhibition by wortmannin, LY294002 and rapamycin of p70 S6 kinase activity stimulated by TSH, forskolin, EGF, HGF and PMA suggested that PI 3-kinase(s) and mTOR reside in the pathways controlled by all these agents leading to p70 S6 kinase stimulation. Moreover, mTOR and PI 3-kinase seem to be necessary to induce the transition of quiescent cells through G_1 into S phase as rapamycin and wortmannin, even added late in G_1 , induced a marked decrease of the [3 H]thymidine incorporation into nuclei and the prevention of the hyperphosphorylation of pRb and its related family members p107 and p130. As mTOR has been shown to have other substrates than p70 S6 kinase [40,41], we cannot confirm that p70 S6 kinase plays a necessary role in the proliferation of dog thyrocytes, but our results are compatible with this idea. On the other hand, as neither insulin, nor TSH, forskolin, EGF or PMA is mitogenic *per se*, activation of p70 S6 kinase alone is certainly not sufficient to trigger DNA synthesis.

In order to investigate the role of PI 3-kinase(s) in the activation of p70 S6 kinase and/or DNA synthesis by the different mitogens acting on thyrocytes, we have studied the effect of these agents on the formation of 3-phosphoinositides products and on the activity of PI 3-kinase and of PKB. As already suggested by its mechanism of activation [10], PKB has been shown to be a target not only for the much studied PI 3-kinases α and β but also for PI 3-kinase γ [42]. Its activation is therefore a good marker of the activation of any known isoform of PI 3-kinase [10]. In our work, we show that all of the growth factors tested which stimulate cells via receptor tyrosine kinases (insulin/IGF-1, HGF and EGF) were able to provoke the appearance of 3-phosphorylated inositol lipids in intact cells, the association of a PI 3-kinase with anti-phosphotyrosine antibodies and the phosphorylation and activation of PKB. Moreover there is a parallelism in the efficacy of these different agents to stimulate PI 3-kinase and PKB, insulin and HGF causing a strong and sustained activation of these enzymes whereas the effect of EGF on these activities was weak and transient. The inability of TSH, forskolin or PMA to cause the formation of $\text{PtdIns}(3,4,5)\text{P}_3$ in intact cells and the activation of its direct target PKB strongly suggest that, in dog thyroid cells, TSH and cAMP as well as protein kinase C are not coupled to the formation of 3-phosphoinositides. Thus in dog thyrocytes, the events leading to p70 S6 kinase activation following exposure of the cells to TSH or PMA participate in pathways which do not include PI 3-kinase and which are still unknown.

The inhibition by wortmannin and LY294002 of the TSH (or forskolin) or PMA stimulated p70 S6 kinase activity is intriguing. It may suggest a lack of specificity in the action of these drugs. Indeed wortmannin and LY294002 have been shown to suppress the kinase activity of mTOR *in vitro* [43] and the former to inhibit stimulated phospholipase A_2 activity in Swiss 3T3 cells [44].

A lack of PI 3-kinase/PKB activation by phorbol esters has already been shown in other systems [42,45], while there are only few data concerning the regulation of these kinases by G-protein-coupled receptors. Recently Murga et al. [42] have reported that the pathway connecting G proteins to PI 3-kinase and PKB implicates a signal emanating from $G\alpha_q$, $G\alpha_i$, and $\beta\gamma$ dimers, but not from $G\alpha_{12}$ or $G\alpha_s$. This is in agreement with the fact that TSH and its receptor which act, in dog thyroid cells, mostly through $G\alpha_s$ [46] is without effect on these enzymes. However in the WRT cell line, TSH and cAMP-elevating agents were reported to stimulate PKB through a PI 3-kinase dependent pathway [37],

while in COS cells, cAMP-elevating agents stimulate PKB through a PI 3-kinase independent one [13]. Obviously, as for other characteristics, the pathways stimulated by cAMP are different in these cell lines [15].

The fact that EGF, which strongly stimulates p70 S6 kinase, had only a weak and transient effect on PKB and on PI 3-kinase activities, is in agreement with the results obtained in skeletal muscle and adipose tissue [47]. It suggests the implication of other pathways in the action of this agent on p70 S6 kinase activity.

In contrast, we found a perfect correlation between the effect of insulin on PI 3-kinase, PKB and p70 S6 kinase activities which were all strongly stimulated by this agent. These observations are in agreement with numerous reports which implicate PI 3-kinase and PKB in the activation of p70 S6 kinase by insulin [10]; they do not shed light on the exact architecture of the pathway leading to p70 S6 kinase activation which is still unknown. HGF, the only growth factor acting on thyroid cells which does not require the presence of insulin to induce DNA synthesis, also strongly stimulates PI 3-kinase/PKB and p70 S6 kinase activities. Unlike the activation of p70 S6 kinase, the activation of the PI 3-kinase/PKB pathway thus represents the first effect of insulin not reproduced by the mitogenic agents which require insulin action. It is an obvious candidate to explain the permissive role of this hormone in the mitogenic effect of TSH, PMA and perhaps EGF in dog thyroid cells. Several facts support this hypothesis: (i) HGF, the only mitogenic agent which does not require insulin for its action, strongly activates PI 3-kinase and PKB, (ii) DNA synthesis induced by HGF in the absence of insulin is strongly inhibited by wortmannin, (iii) PMA and EGF, which as insulin activate the MAPK/p90 S6 kinase pathway but do not or weakly activate PI 3-kinase, are not permissive agents for TSH or forskolin [17]. However in the case of the mitogenesis induced by TSH or forskolin we cannot exclude a complementary contribution of the p42/p44 MAPK pathway in the permissive effect of insulin. As insulin is routinely added to many cell culture media, this permissive mechanism may in fact apply to other cell types. Moreover, contrary to what was reported in the WRT cell line [37], we demonstrate in the physiologically relevant system of primary cultures of dog thyroid cells, that as PI 3-kinase/PKB activity is not enhanced by the physiological stimulus of thyroid cells cAMP, the PI 3-kinase/PKB pathway cannot account either for the PKA-dependent mitogenic pathway(s) or for the PKA-independent one(s). Finally, as insulin/IGF-1 greatly activates the PI 3-kinase/PKB pathway but does not by itself induce mitogenesis or dedifferentiation, the activation of this pathway *per se* is not sufficient to elicit these processes in thyroid cells.

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