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Transmembrane signalling pathways initiating cell growth in fibroblasts

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The mechanisms of growth factor action were studied in a fibroblastic cell line capable of reversible growth arrest in G0–G1. This cell line, derived from Chinese hamster lung, can be stimulated to divide by a limited set of purified growth factors, including EGF, FGF, PDGF, α-thrombin (THR), serotonin (5-HT) and insulin. THR and 5-HT stimulate, via a G-protein (G_p), a polyphosphoinositide-specific phospholipase C (PtdIns(4,5)P_2–PLC). In contrast, the mitogens EGF, FGF, PDGF, and insulin do not stimulate PtdIns(4,5)P_2–PLC unless this pathway has been preactivated by THR or AlF_4^-. Finally, from the specific inhibitory action of pertussis toxin on THR- and 5-HT-induced DNA synthesis, and from the exploitation of the 5-HT pharmacological tools, we conclude that: (i) there are at least two distinct G-proteins involved in signalling growth: G_p, coupling receptors to PtdIns(4,5)P_2–PLC, and G_i, coupling receptors negatively to adenylyl cyclase and probably to other unknown effector(s); (ii) activation of receptor-tyrosine kinases provides an alternate growth factor signalling pathway, independent of G_p- and G_i-mediated actions; and (iii) tyrosine kinases positively 'cross-communicate' with the inositol-lipid pathway (phosphorylation of G_p, PLC, PtdIns kinases...?).

Introduction

Growth factors and hormones play a central role in growth and differentiation of multicellular organisms. In general, a given hormone elicits several biological responses. This multiplicity of action results from the complex communication between cells of different embryological origin and from the fact that a given hormone can interact with a subset of receptors, differentially expressed and engaged in distinct signal-transducing pathways. Serotonin (5-HT), and its interaction with at least six subtypes of receptor, provides a fascinating example of biological complexity (Fozard 1987).

Therefore, studies with cells of clonal origin and performed with chemically defined media have greatly facilitated the analysis of hormone and growth-factor action at a molecular level. In this report, we discuss the nature of the signalling pathways which might be directly involved in the control of fibroblast cell proliferation. In particular, we bring a critical view to the new and rapidly expanding idea that the breakdown of inositol lipids plays a crucial role in the control of growth.

We shall restrict our discussion to the mode of action of growth factors which, either alone or in association, are capable of recruiting G0 resting cells into the phase of DNA replication. The studies presented rest on the use of a diploid cell line (CCL39) of Chinese hamster lung fibroblasts (CHL) capable of reversible arrest in G0 (Pouysségur *et al.* 1980), amenable to genetic analysis (Pouysségur & Franchi 1986) and in which growth factor relaxation leading

to oncogenesis has been analysed in some detail (Pérez-Rodriguez *et al.* 1981*b*; Renwick *et al.* 1986). Also, to avoid some of the problems inherent in the use of an immortal cell line, the generality of our conclusions has been verified on secondary cultures of CHL cells.

Growth factors for Chinese hamster lung fibroblasts

CHL fibroblasts, from the cell line CCL39 or secondary cultures, grow with a generation time of 12–15 h in medium supplemented with 10% foetal calf serum. Complete removal of serum for 24 h is sufficient to arrest the entire population (over 99%) in a post-mitosis resting state referred to as G0–G1. With the sole presence of insulin (10 $\mu g\ ml^{-1}$) and transferrin, these quiescent cells remain viable for at least a week, as judged by their ability to re-enter the proliferative state in response to serum. A limited set of pure hormones or growth factors elicited reinitiation of DNA synthesis in these G0-arrested CHL cells. In decreasing order of potency, we have: α-thrombin (THR), fibroblast growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), 5-HT and insulin (substituting for insulin-like growth factor I, IGF-I). For instance, at their optimal concentrations and depending on whether CCL39 or secondary cultures are used, THR, FGF, or EGF alone trigger DNA replication in 30–40%, 10–25% and 5–20% of the cells, respectively, as measured by labelled nuclei. Insulin (IGF-I) has no mitogenic action *per se* but potentiates the mitogenic action of all other growth factors. 5-HT, a weak mitogen, potentiates the action of EGF, PDGF and especially that of FGF. Interestingly, phorbol esters added at different concentrations, either alone or in combination with one or two of the above-described mitogens, failed to stimulate DNA synthesis.

Another point of interest is that these mitogens are also competent for promoting continuous cell proliferation of CCL39 cells in serum-free medium (Pérez-Rodriguez *et al.* 1981*a*). The minimal combination, insulin and THR (1 nM) triggers a sustained growth rate with a generation time of 15–18 h (Van Obberghen-Schilling & Pouysségur 1983).

THR, FGF, and EGF trigger a common set of early mitogenic events

Over the past five years it has become evident that an increasing list of growth-promoting agents acting on different types of cells elicit a common pleiotypic response (Rozengurt 1986). For instance, THR, FGF, and EGF, the three distinct growth-promoting agents for CCL39, acting on different cell-surface receptors, elicit in common: (i) a rise in intracellular pH owing to activation of the amiloride-sensitive Na^+–H^+ antiporter (Pouysségur 1985; Magnaldo *et al.* 1986); (ii) a transient rise in cytoplasmic Ca^{2+} (Magnaldo *et al.* 1986); (iii) the phosphorylation of a common set of proteins, ribosomal protein S6 (Chambard *et al.* 1983), the 27 kDa stress protein (Chambard *et al.* 1983; Welch 1985) and the 41 and 43 kDa proteins (Kohno & Pouysségur 1986); (iv) the increase in c-*fos* and c-*myc* mRNA (Blanchard *et al.* 1985; Magnaldo *et al.* 1986). Although the precise role of these biochemical changes is still poorly understood, their conservation between species, and the commonness of activation, is the strongest indication that they might play a determinant role in progression from G0–G1 to S-phase. Another indication is that, for mitogenicity, the persistence of these 'activated' events requires persistent occupation of thrombin receptors (Van Obberghen-Schilling *et al.* 1985).

The crucial question for our discussion is whether THR, EGF and FGF activate distinct

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transmembrane signalling pathways, or share a common one. In the absence of a common pathway, is there an early convergent step inducing a unifying response to such a diversity of external stimuli?

THR AND 5-HT ACTIVATE INOSITOL LIPID BREAKDOWN, BUT PDGF, EGF, FGF AND IGF-I DO NOT

Receptor-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) as a source of second-messenger molecules is now widely established (Berridge 1984; Nishizuka 1986). Inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) releases Ca^{2+} from intracellular stores, and diacylglycerol (DG) activates protein kinase C (PKC).

It was of interest to see that THR, the most potent of the mitogens for quiescent CHL fibroblasts, potently induces inositol lipid breakdown. The time course of thrombin-induced inositol phosphate production is shown in figure 1. Ins(1,4,5)P_3 could be detected as early as 5 s after thrombin addition which somewhat parallels a transient rise in Ca^{2+} (figure 1, bottom). Interruption of the PtdIns cycle with Li^+, illustrates the massive PtdIns hydrolysis generated by an optimal mitogenic concentration of THR (figure 1, inset). 5-HT at 10 μM is a

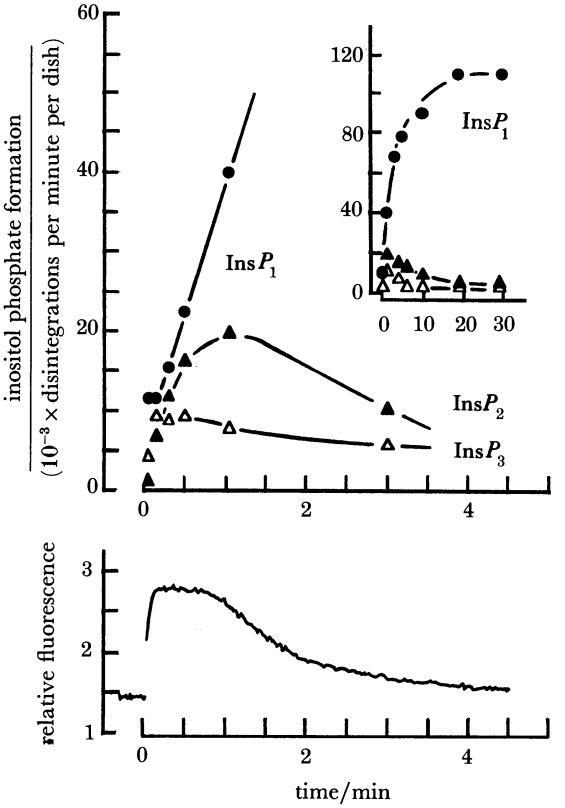

FIGURE 1. Kinetics of inositol phosphate formation and rise of Ca_i^{2+} after thrombin addition. CCL39 cells were grown to confluency in medium containing foetal calf serum (10% by volume), arrested in G0/G1 and prelabelled with [^{3}H]inositol as previously described (Paris & Pouysségur 1986). Top, cells were transferred into HEPES-buffered medium, Li^+ (20 mM) was added and 10 min later ($t = 0$) 1 nM THR; bottom, parallel cultures of CCL39 cells grown on glass coverslips and arrested in G0/G1 were incubated with Quin2 AM for 30 min. 1 nM THR was added at time 0 and fluorescence recorded as described (Magnaldo *et al.* 1986).

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weak mitogen when added alone to quiescent CHL cells, but it significantly induces PtdIns hydrolysis. The very low rate of basal PtdIns(4,5)P_2–PLC activity of quiescent CHL cells, is stimulated 5- to 10-fold by 5-HT. However, this 5-HT-induced PLC activation represents only about one tenth of that induced maximally by THR. Another point of interest for the rest of the discussion is that ketanserin, a blocker of 5-HT_2 receptors (Fozard 1987), abolishes 95% of the 5-HT-induced PLC activation (Seuwen *et al.* 1988).

Similar to the human neutrophil system, as first reported by Cockroft & Gomperts (1985), hormonal activation of PtdIns(4,5)P_2–PLC in CHL fibroblasts is mediated via a GTP-binding protein. The first indication is that both THR- and 5-HT-induced inositol phosphate are inhibited (50%) by pretreating the cells with the toxin of *Bordetella pertussis* (Paris & Pouysségur 1986; Seuwen *et al.* 1988). A more direct indication in favour of a G-protein in the process of PLC activation relies on the fact that AlF_4^-, a general 'agonist' of G-proteins (Gilman 1984; Bigay *et al.* 1985), activates PLC in intact CCL39 cells (Paris & Pouysségur 1987) and that CCL39 membranes release Ins(1,4,5)P_3 in response to THR only in the presence of a GTP analogue (Magnaldo *et al.* 1987).

Finally, with CHL quiescent cells (CCL39 or secondary cultures) and Li^+ to amplify the sensitivity of the assay, the other pure mitogens tested, acidic and basic bovine FGF, mouse EGF, human PDGF and insulin (IGF-I) did not induce a significant accumulation of inositol phosphate (L'Allemain & Pouysségur 1986; Paris *et al.* 1988). Even a potent mitogenic combination like EGF and insulin, for arrested CHL cells, failed to stimulate PtdIns(4,5)P_2–PLC at early, mid and late G1 phase, whereas PLC in these cells remained highly activatable by THR (L'Allemain & Pouysségur 1986). If inositol lipid breakdown plays a role in signalling cell replication (see discussion below) it was evident to us that this pathway was not an absolute prerequisite for growth. It is noteworthy that all these mitogens, which apparently are not directly coupled to PLC activation, recognize receptor-tyrosine kinases (Hunter & Cooper 1985), with the exception of FGF for which some uncertainty remains. Elegant studies, in which engineered point mutations have abolished the tyrosine kinase functional domains of insulin receptors (McClain *et al.* 1987) and EGF receptors (Chen *et al.* 1987; Livneh *et al.* 1987), strongly support the view that transmembrane activation of the tyrosine kinases suffices to generate all biological effects.

FGF, EGF AND PDGF POTENTIATE THE ACTIVATION OF PLC: AN EXAMPLE OF POSITIVE CROSS-COMMUNICATION

Because association of mitogens very often leads to synergistic effects rather than additivity in DNA synthesis (Rozengurt 1986), it was of interest to analyse whether potentiation could take place at the level of transmembrane signalling. The first example we examined was the very potent association of FGF and THR. When added to G0-arrested CCL39 cells, FGF (100 ng ml^{-1}) did not significantly modify either the low basal or the THR-induced rates of inositol phosphate release. However, if FGF is added after THR-activated PLC has reached the steady state that follows THR-receptor desensitization, then FGF potentiates PLC activation (figure 2) (Paris *et al.* 1988). This potentiation, up to twofold, is not due to a partial reversal of THR-receptor desensitization, since it is also observed if PLC is activated by AlF_4^-, an action which is not receptor-mediated. It is not mimicked by calcium ionophores, but is likewise elicited by EGF and PDGF and to a lesser extent by insulin.

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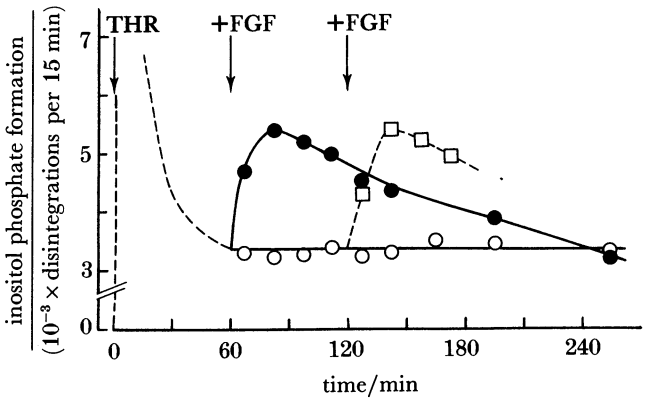

FIGURE 2. Potentiation by FGF of the THR-induced inositol phosphate release. Quiescent [^{3}H]inositol-labeled cells were stimulated at time 0 with 1 nM THR. Some cultures received in addition 100 ng ml^{-1} FGF either 60 min (●) or 120 min (□) after THR addition. 20 mM LiCl was added at various times and linear accumulation of inositol phosphate was measured over 15 or 30 min. PLC activity values, measured by the rate of Li^+-induced accumulation of inositol phosphate, are plotted at the times corresponding to the middle of Li^+ pulse.

These observations deserve some discussion. Whereas a consensus can be easily reached concerning PLC activation by a set of mitogens, including bombesin, bradykinin, vasopressin, thrombin and serotonin, PLC activation by PDGF, EGF and FGF remains controversial. EGF stimulates the formation of inositol phosphates in A431 cells (Wahl *et al.* 1987; Pike & Eakes 1987) and hepatocytes (Johnson & Garrison 1987) but not in 3T3 or CHL fibroblasts (L'Allemain & Pouysségur 1986; Besterman *et al.* 1986). FGF stimulates PtdIns breakdown and PKC in Swiss 3T3 but not in CHL cells (Tsuda *et al.* 1985; Magnaldo *et al.* 1986). The final, and perhaps the most disturbing, aspect is the case of PDGF. This has been reported to activate PLC, although weakly, in all the systems so far studied; for example, human fibroblasts (Chu *et al.* 1985), mouse Balb/c 3T3 (Besterman *et al.* 1986) and Swiss 3T3 fibroblasts (Berridge *et al.* 1984). Our interpretation of the present observations, and this apparent contradiction, has led us to propose the following two conclusions.

(i) Tyrosine kinase receptors for EGF, PDGF, insulin, IGF-I (and FGF?) cannot directly activate 'resting' PtdIns(4,5)P_2–PLC, a process specifically requiring dissociation of a G-protein (G_p).

(ii) If G_p is partly dissociated by some 'leaky or autocrine' mechanism, or by addition of an agonist, activated receptor tyrosine kinases do potentiate hydrolysis of polyphosphoinositides.

These conclusions are inspired by the fact that EGF-, FGF-, and PDGF-induced PLC activation are conditional to pre-activation of PLC via a G-protein mediated process (THR or AlF_4^-) and the fact that receptors coupled to G-proteins form a conserved family (Dohlman *et al.* 1987) that is strikingly distinct from the structure of tyrosine kinase receptors (Ullrich *et al.* 1986). The contradictions reported in the literature can be easily explained if we imply that the differences in the sensitivity of PtdIns breakdown to tyrosine kinase-activating agents, reflect primarily different 'basal' PLC activities linked to a variable capacity of cell types to quiesce 'deeply' in G0. This sensitivity would also be magnified by different levels of tyrosine kinase receptors (Wahl *et al.* 1987).

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Evidence for independent growth-factor signalling pathways

If we postulate that PLC activation plays a determinant role for mediating the mitogenic action of THR, it is an easily testable prediction that pertussis toxin, an inhibitor of THR-induced inositol lipid breakdown, should attenuate THR-induced mitogenicity. A second prediction is that growth factors (EGF, PDGF, IGF-I and possibly FGF), which activate receptor-tyrosine kinases via transmembrane signalling pathways not coupled to inositol lipid breakdown, should not be affected by the toxin. The experiments with G0-arrested CCL39 or CHL cells depicted in figure 3 entirely satisfied these two predictions (Chambard *et al.* 1987). THR-induced reinitiation of DNA synthesis of CCL39 or CHL cells is antagonized up to 95%

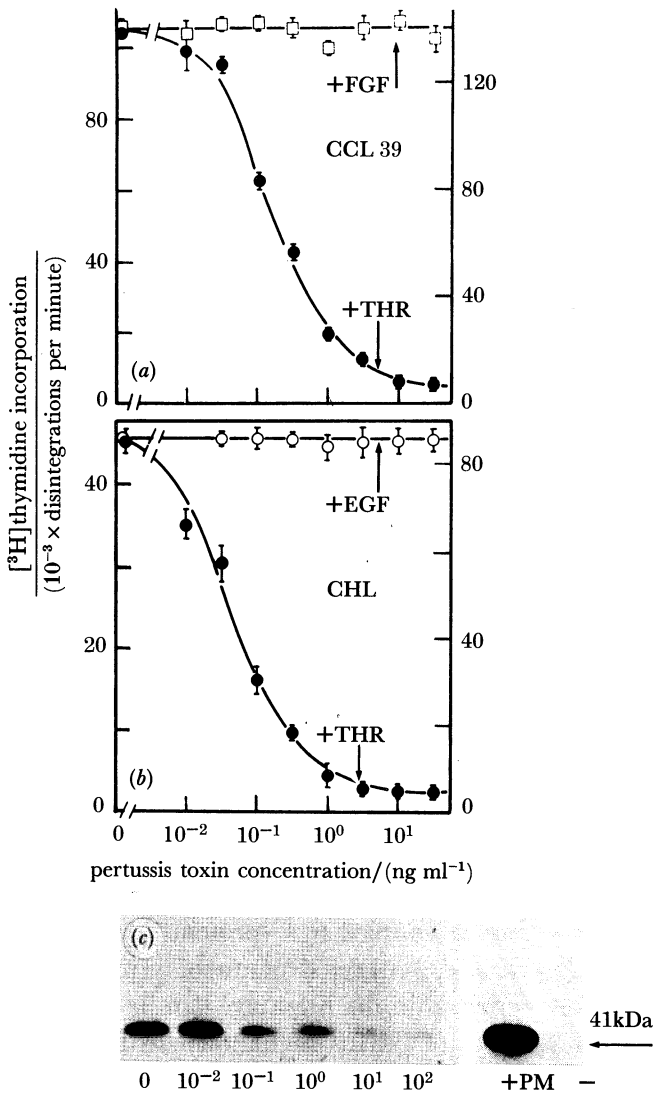

Figure 3. Parallel effect of pertussis toxin on THR-, EGF- and FGF-induced reinitiation of DNA synthesis and on ADP-ribosylation *in vivo*. G0-arrested cultures of CCL39 (*a*) or secondary cultures of CHL fibroblasts (*b*) were preincubated for 4 h with [³H]thymidine and the indicated concentrations of pertussis toxin. Incubation was continued for 24 h in either 1 nM THR (●), 100 ng ml^{-1} basic FGF (□) or 50 ng ml^{-1} EGF (○). (*c*) Dose–response of toxin-catalysed *in vivo* ADP-ribosylation (reprinted from Chambard *et al.* 1987).

by 10 ng ml^{-1} of pertussis toxin. Parallel cultures reinitiated either by EGF or FGF were not affected by the toxin at concentrations up to 100 ng ml^{-1}. The dose–response curve of toxin-induced ADP-ribosylation of a 40–41 kDa protein *in vivo*, parallels toxin-induced inhibition of DNA synthesis (figure 3, bottom). A more precise analysis of the pertussis toxin substrates in these cells has indicated the presence of two discrete G_α subunits of 41 and 40 kDa (B. Rouot, personal communication) corresponding to $G_{i\alpha}$ and $G_{N\alpha}$ ($G_{40\alpha}$ of neutrophils) as determined by specific immunoblotting (P. Gierschik, personal communication). These results clearly indicate: (1) the existence of at least two separate growth-factor-signalling pathways capable of operating independently; and (2) that one or two G-proteins, substrates of pertussis toxin, participate in the initiation of growth by THR. These results were first interpreted by us as a strong indication that inositol lipid breakdown plays a crucial, although not essential, role in dictating cells to replicate DNA (Chambard *et al.* 1987). However, this conclusion needs some re-evaluation. The issue is complicated; first, by the fact that pertussis toxin antagonizes only 50%, at most, of THR-activated PLC; secondly, because we cannot be certain whether G_p is a direct substrate for the toxin; and thirdly, THR, like 5-HT, activates other pertussis toxin-sensitive signalling pathways (see next section).

A novel G-protein-mediated growth signalling pathway, sensitive to pertussis toxin

Inhibition of THR-induced DNA synthesis by pertussis toxin appears more drastic than inhibition of THR-induced IP release. Indeed, pertussis toxin, which attenuated THR-activated PLC by 50% at all THR concentrations tested (Paris & Pouysségur 1986) shifted the THR dose–response curve for reinitiation of DNA synthesis by 2–3 orders of magnitude to the right. This was the first observation suggesting that besides inositol lipid breakdown, another growth-signalling pathway, sensitive to pertussis toxin, must operate at low THR concentrations.

We were able to confirm and extend this observation to the mitogenic action of 5-HT. This was a key finding because the 5-HT receptor pharmacology is far more advanced than that of THR, for which even the concept of a receptor is still a matter of controversy. Figure 4 shows that 5-HT is not mitogenic on its own, but significantly enhances the mitogenicity of EGF, and especially that of FGF. Note that THR-induced mitogenicity is not affected by 5-HT, a result expected from mitogens of the same 'family'. Pertussis toxin completely abolished 5-HT-induced mitogenicity, leaving intact the FGF response (figure 4). In contrast, ketanserin, a 5-HT_2 receptor blocker (as shown by the 95% inhibition of 5-HT-induced inositol phosphate release), did not prevent 5-HT-induced mitogenicity (Seuwen *et al.* 1988). This result clearly demonstrates that the mitogenicity of 5-HT is not supported by its action on inositol lipid breakdown, and illuminates a novel mitogenic-signalling pathway, that is sensitive to pertussis toxin. This pathway might be the same as is operating in response to low THR concentrations or to 5-HT in smooth muscle cells (Kavanaugh *et al.* 1988) or to bombesin in 3T3 mouse fibroblasts (Zachary *et al.* 1987).

What is the nature of this signalling pathway and which G-protein is associated with it? Besides activation of PLC, THR and 5-HT inhibit in common adenylyl cyclase (AC) in CHL fibroblasts. This action, which is best measured on hormonally activated AC, involves a receptor-mediated dissociation of G_i, a step completely inhibited by pertussis toxin. The 5-HT

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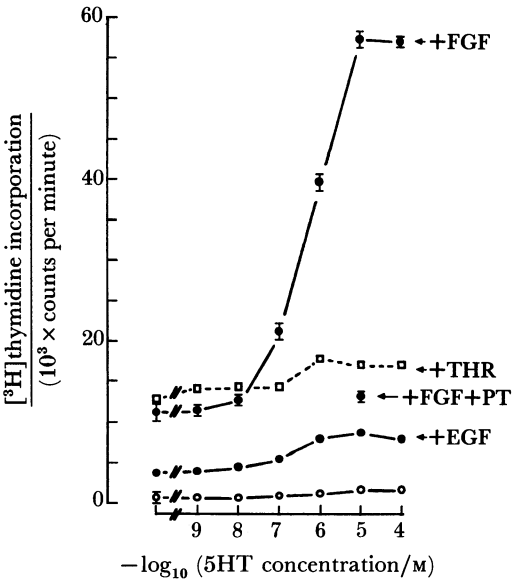

FIGURE 4. Concentration-dependence of 5-HT-induced DNA synthesis. Effects of EGF, FGF, and pertussis toxin. The experiment was done in G0-arrested CCL39 cells. THR was added at 1 nM, EGF at 50 ng ml^{-1}, FGF at 100 ng ml^{-1} and pertussis toxin (PT) at 20 ng ml^{-1}.

pharmacological tools were decisive in assigning a crucial role of this G_i-coupled pathway in mitogenesis. A specific antagonist of the 5-HT_{1B} receptor subtype suppressed 5-HT-mediated AC inhibition and, most importantly, suppressed 5-HT-induced mitogenic action. This finding, and the fact that a 5-HT_{1B} agonist restores mitogenicity without activating PLC, reinforces the specificity of this receptor-mediated G_i action (Seuwen *et al.* 1988).

Now, a crucial question is to know whether $G_{i\alpha}$-mediated inhibition of AC is the triggering event of the 5-HT-induced mitogenic response and/or whether $G_{i\alpha}$ is coupled to some other effector that remains to be discovered. Currently, we are testing the hypothesis according to which a sustained decreased basal level of cAMP could mimic 5-HT mitogenic action. This hypothesis is certainly consistent with the inhibition of CHL-induced DNA synthesis by agents increasing cAMP levels. However, we prefer the second hypothesis in which $G_{i\alpha}$ is coupled to other effectors. Our present working hypothesis is that $G_{i\alpha}$ is coupled in a direct or indirect fashion to a tyrosine kinase activity, c-src or members of its family being just favourite candidates.

CONCLUSIONS

We have analysed and discussed in this chapter only some limited aspects of the events controlling cell proliferation. Although we asked a simple question – which of the multiple membrane signal-transducing mechanisms play a direct role in dictating the replication of DNA in resting cells – the answer has not been so easy and is still incomplete. In particular, three sources of complications could lead to misinterpretations and generate some controversy in this issue: (1) basal activity of membrane receptor-coupled enzymes varies from one cell type to another; (2) a complex network of positive and negative interactions exists between membrane-signalling pathways; and (3) often a hormone interacts with more than one population of receptors in a given cell, so superimposing the effects of multiple-signalling pathways.

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Despite these difficulties, the following few conclusions can be drawn: (1) an important new class of growth factors is emerging, consisting of neurohormones, neurotransmitters, neurodigestive peptides and vasoconstrictors (thrombin, serotonin, bradykinin, bombesin, vasopressin, substance P, etc); (2) their actions appear to be mediated through multiple G protein-coupled processes; (3) there are at least two G-proteins involved in signalling growth: G_p, a G-protein coupled positively to PtdIns(4,5)P_2–PLC; and G_i, a G-protein coupled negatively to AC and very likely to other unknown effectors important for growth; (4) receptor-tyrosine kinases for EGF, PDGF, IGF-I (and maybe FGF) provide alternative growth-signalling pathways independent of G_p- and G_i-mediated actions; (5) tyrosine kinases cross-communicate with the inositol lipid pathway by a mechanism that could imply phosphorylation of G_p, PLC or PtdIns-kinases.

Obviously more work is needed to confirm these points and to elucidate the key targets and the sequence of events participating in the onset of DNA replication.

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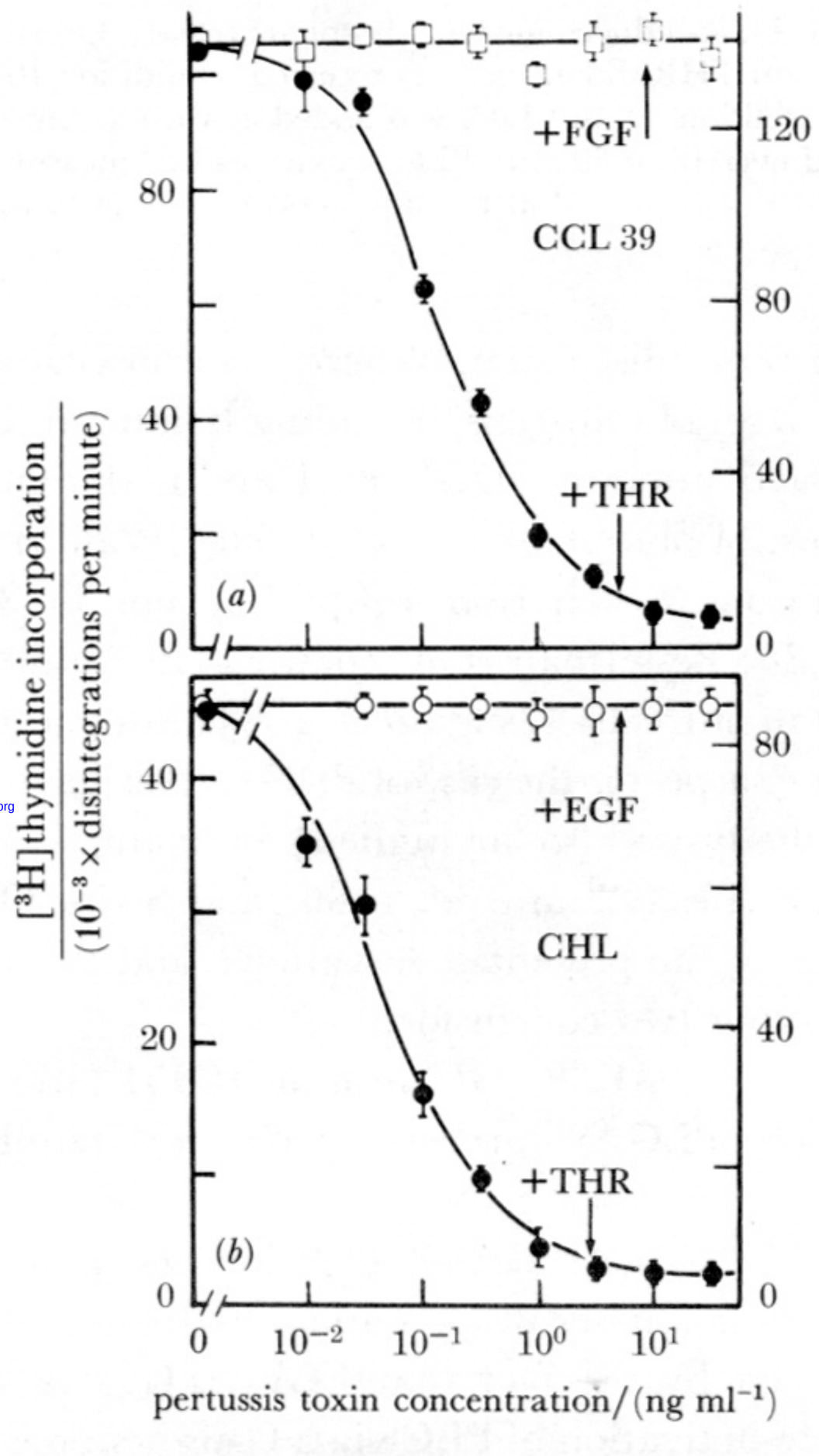

FIGURE 3. Parallel effect of pertussis toxin on THR-, EGF- and FGF-induced reinitiation of DNA synthesis and on ADP-ribosylation *in vivo*. G0-arrested cultures of CCL39 (*a*) or secondary cultures of CHL fibroblasts (*b*) were preincubated for 4 h with [³H]thymidine and the indicated concentrations of pertussis toxin. Incubation was continued for 24 h in either 1 nM THR (●), 100 ng ml^{-1} basic FGF (□) or 50 ng ml^{-1} EGF (○). (*c*) Dose–response of toxin-catalysed *in vivo* ADP-ribosylation (reprinted from Chambard *et al.* 1987).