0013-7227/01/$03.00/0
Endocrinology

Vol. 142, No. 3
Printed in U.S.A.

Respective Roles of Carbamylcholine and Cyclic Adenosine Monophosphate in Their Synergistic Regulation of Cell Cycle in Thyroid Primary Cultures*

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ABSTRACT

The stimulation of thyroid cell proliferation by TSH through cAMP depends on permissive comitogenic factors, generally the insulin-like growth factors and insulin. In dog thyroid primary cultures, the use of the phosphodiesterase-resistant analog of cAMP $(Bu)_2$cAMP instead of TSH allowed to unveil a potent comitogenic activity of carbamylcholine, which can substitute for insulin and was shown to mimic insulin action on cell cycle regulatory proteins. Like insulin, carbamylcholine induced the accumulation of cyclin D3 and overcame the repression by cAMP of this protein, which was shown 1) to be essential for cell cycle progression by means of microinjections of a neutralizing antibody; and 2) to be rate limiting for the cAMP-dependent assembly of cyclin D3-cdk4 complexes, their nuclear translocation and the phosphorylation of pRb. Relative to insulin, carbamylcholine offers the significant experimental advantage that its signaling cascades can be immediately deactivated by the muscarinic antagonist atropine. In the presence of carbamylcholine, the elimination of $(Bu)_2$cAMP blocked within 2 h the entry of cells into DNA synthesis phase, but the addition of atropine still permitted the entry of cells in S phase. These data support our view that the progression in G1 phase stimulated by cAMP consists of at least two essential actions that are clearly dissociated: in a first stage, depending on the supportive activity of an agent that stimulates the required cyclin D3 accumulation, cAMP induces the assembly and nuclear translocation of cyclin D3-cdk4 complexes, and then cAMP can exert alone the last crucial control that determines the cell commitment toward DNA replication. (*Endocrinology* **142:** 1251–1259, 2001)

DOG THYROID epithelial cells in primary culture constitute a model of positive control of DNA synthesis initiation and G0-S prereplicative phase progression by cAMP as a second messenger for TSH (1). In this system, as well as in human thyrocytes and in the FRTL-5 rat thyroid cell line, the stimulation of DNA synthesis and proliferation by TSH depends on the presence of IGF-1 (insulin-like growth factor 1) or insulin (2–4). As the real physiological stimulator of thyroid cells is TSH, the comitogenic role of insulin or IGF-1 is described as permissive for the proliferative action of TSH in dog and human thyrocytes (5). However, in various *in vitro* thyroid models the respective roles of TSH and insulin/IGF-1 are a matter of major controversy. A central question is whether TSH and insulin/IGF-1 through distinct signaling cascades exert complementary functions required for cell proliferation, or whether one of these factors exerts priming actions making the cell more competent to respond to the other one.

In dog thyrocytes primary culture, the roles of TSH (cAMP) and insulin/IGF-1 on cell cycle progression have been recently found to be distinct and complementary (6). Their signaling pathways are largely independent. TSH, unlike insulin/IGF-1 and/or growth factors, does not activate the Ras/MAP kinases pathway nor the PI3 kinase/PKB pathway (7–9). Insulin/IGF-1 but not TSH induces the increase of cell mass (hypertrophy) required for repetitive cell divisions (10). Through a novel but still partly unclear mechanism, the comitogenic effects of TSH and insulin on G1 phase progression and DNA synthesis initiation are integrated with the cell cycle machinery at the level of the activation of cyclin D3-cdk4 (cyclin-dependent kinase 4) (6), resulting in the phosphorylation of pRb and related p107 and p130 (11), the activation of cdk2, and the E2F-dependent transcription of genes such as cyclin A and cdc2 (12). Indeed, we have shown that cyclin D3, which is the most abundant cyclin D in dog thyrocytes, and cdk4 are required for entry into S phase of cells stimulated by TSH in the presence of insulin (13). TSH alone paradoxically inhibits the basal accumulation of cyclin D3, but insulin stimulates it, thus overriding the inhibitory effect of TSH (6). TSH increases the accessibility of a cyclin D3 epitope and induces the formation and nuclear translocation of cyclin D3-cdk4 complexes in the presence of insulin, whereas insulin alone fails to do so (6). When both hormones are present simultaneously, they can thus complement each other; insulin permits the activation of cdk4 by TSH by sup-

Received September 7, 2000.

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* This study was supported by the Belgium Program on University Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy programming, and by grants from the National Fund for Scientific Research (FNRS, Belgium), the Belgian Fund for Scientific Medical Research (FRSM), the Caisse Générale d'Epargne et de Retraite, the Télévie, the Association Belge contre le Cancer and the Association Sportive contre le Cancer.

† Fellow of the fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA).

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plying the required cyclin D3, which is activated by TSH and cAMP, allowing the formation and translocation of cyclin D3-cdk4 complexes (6).

Carbamylcholine (carbachol) has long been demonstrated as an important modulator of thyroid cell function, including in dog thyrocytes (14, 15). Through muscarinic receptors coupled to phospholipase C, it stimulates the iodination of thyroglobulin through increased H_2O_2 generation (16) and apical iodide efflux (17), but it decreases the accumulation of cAMP induced by TSH (18) and thyroid hormone secretion (15). Recently, we have observed that carbachol induces the hypertrophy of thyrocytes, as does insulin (10). It also induces several so called early mitogenic events such as the activation of MAP kinases (7) and the expressions of c-*fos*, c-*myc*, and *egr-1* (19–21). However, it is unable to trigger DNA synthesis, alone or in the presence of insulin (20). Here we report our observation that carbachol can replace insulin and permit the induction of DNA synthesis by the nonhydrolysable cAMP analog $(Bu)_2$cAMP. By the analysis of cell cycle regulatory proteins, we identify cyclin D3 accumulation as the convergence point of the additive permissive effects of carbachol and insulin. Moreover, we exploit the possibility to immediately deactivate the signaling cascades of carbachol by the administration of atropine to address the crucial question of the stages of G1 phase that depend on permissive factors or cAMP.

Materials and Methods

Primary cultures of dog thyroid follicular cells

Dog thyrocytes, seeded as follicles (2×10^4 cells/cm^2), were cultured in monolayer in the following mixture (22): DMEM + Ham's F12 medium + MCDB104 medium (2:1:1, by volume; Life Technologies, Inc., Paisley, Scotland, UK), supplemented with ascorbic acid (40 μg/ml) and antibiotics. The medium was changed every 2 days. At day 4, the cells were quiescent and were treated with the following stimulants: bovine TSH [Sigma (St. Louis, MO), 1 mU/ml), $(Bu)_2$cAMP (Sigma, 10^{-4} M), bovine insulin (Sigma, 5 μg/ml) and carbachol (Sigma, 10^{-5} M). Atropine (Eurobiochem) was used at 10^{-5} M and cycloheximide at 10 μg/ml.

Nomenclature of cell treatments

In figures, the pretreatment of cells applied at day 1 is indicated before the dash, and the stimulation at day 4 by various combinations of factors is indicated after. The following abbreviations are used: α, none; cA, $(Bu)_2$cAMP; Cch, carbachol; i, insulin; T, TSH; At, atropine. Thus (Cch-CchcA) means that the cells were pretreated with carbachol and then stimulated at day 4 by $(Bu)_2$cAMP in the presence of carbachol.

Antibodies

Mouse monoclonal antibodies to cyclin D1 [DCS-6 (23)], cyclin D2[DCS-3 (23)] and cyclin D3 [DCS-22 and DCS-29 (24)] were characterized previously. DCS-31 and DCS-35 are mouse monoclonal antibodies generated upon immunization of BALB/c mice with bacterially produced human cdk4 (13). Anti-cdc2 antibody is a mouse monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-cdk4 and anti-$p27^{kip1}$ rabbit polyclonal antibodies were obtained from Santa Cruz. JG39 is a rabbit polyclonal antibody to bovine cyclin A developed by Julian Gannon and Tim Hunt (12). pRb (Retinoblastoma protein) was revealed with the rabbit polyclonal C-15 pRb antibody (Santa Cruz Biotechnology, Inc.), which reacts with both hypo and hyperphosphorylated forms of pRb. The phospho-p42/p44 MAP kinase (Thr 202/Tyr 204) monoclonal antibody was from New England Biolabs, Inc. (Beverly, MA).

Gel electrophoresis and immunodetection of proteins

Cell proteins were separated by PAGE and immunodetected after Western blotting as previously described (19). Equal amounts of cell protein were loaded on each lane (15 μg). Secondary antibodies coupled to horseradish peroxidase (Amersham Pharmacia Biotech, Arlington Heights, IL) were used for detection by enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech).

DNA synthesis

In most experiments (as indicated in figure legends), cells in 3-cm Petri dishes were stimulated for 48 h in the presence of 10^{-4} M BrdU (bromodeoxyuridine) and 2.10^{-6} M fluorodeoxycitidine for the last 24 h. Cells were fixed and the incorporation of BrdU into nuclei was revealed by immunofluorescence as described (19). The percentage of BrdU-labeled nuclei was evaluated by counting 1,000 nuclei per dish. Because most stimulated dog thyrocytes enter into DNA synthesis after a prereplicative lag phase of a minimum of 20 h (22) and because the dog thyrocyte division cycle does not exceed 48 h (25), the period of BrdU incubation allows labeling of the first wave of cells that were progressing into cell cycle in response to mitogenic treatments.

Indirect immunofluorescence

Cells in Petri dishes (2×10^4 cells/cm^2) were fixed with 2% paraformaldehyde for 90 sec at 4 C and then with methanol for 10 min at −20 C and permeabilized with 0.1% Triton X-100, before indirect immunofluorescent detection performed exactly as described previously (12, 13).

Percentages of cells in the different phases of cell cycle were determined from the different patterns of PCNA immunofluorescent staining (12, 25) by counting at least 500 cells per dish. As demonstrated in dog thyrocytes, quiescent cells are barely stained. Several hours after stimulation with mitogens, late G1 phase cells display a diffuse but gradually increasing nuclear staining of PCNA, which becomes speckled once cells reach S phase. This reflects the association of PCNA with clusters of DNA replication sites. G2-phase cells are characterized by an intense diffuse labeling of large nuclei (25).

Immunoprecipitation

Twenty hours after stimulation by $(Bu)_2$cAMP in the presence or not of carbachol or insulin, subconfluent cultures of thyrocytes that contain the same number of cells were washed with calcium/magnesium-free PBS and lysed in 1 ml lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% NP-40, 50 mM NaF, 1 mM sodium orthovanadate, DTT, and protease inhibitors (pefablock, leupeptin). The cellular lysate was sonicated twice and then precleared with protein A Sepharose (Amersham Pharmacia Biotech) and then incubated with 2 μg of antibody at 4 C for 3 h (monoclonal antibody against cyclin D3 (DCS-28) linked to protein A Sepharose). After three rinsings, the immune complexes were suspended in SDS lysis buffer, boiled for 4 min, and analyzed on 10% SDS-polyacrylamide gels. The proteins were immunodetected as described above using either the DCS-22 cyclin D3 antibody or the DCS-31 cdk4 antibody.

Microinjection

Thyrocytes were microinjected at day 4 of the culture as described (13) with an affinity-purified monoclonal antibody against cyclin D3 (DCS-29, 6 mg/ml) or a control mouse immunoglobulin. As the capacity to proliferate can differ for each follicle-derived cell cluster, but is generally homogenous within each, one half of each cell cluster was microinjected, the other half being used as a control. The microinjected and nonmicroinjected cells were stimulated by $(Bu)_2$cAMP and carbachol or insulin just after the microinjection. BrdU (10^{-4} M) and fluorodeoxycytidine ($2\ 10^{-6}$ M) were added 16 h later and cells were fixed 48 h after the stimulation with methanol for 10 min at −20 C. Injected cells were identified by biotinylated antimouse antibody followed by Texas Red-coupled streptavidin. BrdU incorporation was then codetected as above using the FITC-coupled anti-BrdU antibody and all the nuclei were counterstained with Hoechst 33342 dye. The fraction of cells entering into DNA synthesis was estimated by the percentage of BrdU-labeled nuclei in microinjected cells *vs.* neighboring noninjected cells.

All the experiments were reproduced at least three times with similar results, except the microinjection experiment which was consistently repeated twice.

Results

Carbachol is a permissive factor for the cAMP-dependent mitogenesis

According to previously published criteriae (1), the starting material of the present experiments consisted of a pure population of naturally quiescent dog thyroid epithelial cells in primary culture, the majority of which have not proliferated *in vitro* during the first 4-days incubation in a serum-free medium supplemented or not with insulin (22). At day 4, cells were then stimulated to proliferate using the different combinations of agents. Insulin (i), TSH (T), or $(Bu)_2$cAMP (cA) alone had almost no effect on the percentage of 5-bromodeoxyuridine (BrdU)-labeled nuclei (Fig. 1A). When apparent, the effect of TSH partly depends on autocrine IGF-1 production by cells (26). The effect of adding both insulin and TSH together was far greater than the sum of the effects of each hormone alone showing the marked synergy between the signaling cascades. This effect was totally reproduced when TSH was replaced by $(Bu)_2$cAMP, a nonhydrolysable analog of cAMP. Carbachol (Cch), alone or in the presence of insulin, had no or marginal effects on the entry of the cells in S phase. When carbachol was used instead of insulin, it did not permit (or weakly in a few experiments) the mitogenesis triggered by TSH, as shown previously (10). However, the combination of carbachol and $(Bu)_2$cAMP triggered the entry in S phase of a large number of cells (Fig. 1A). This effect of carbachol was concentration-dependent and maximum at 10^{-5} M (Fig. 1B). The comitogenic effect of carbachol in the presence of $(Bu)_2$cAMP was consistently reproduced in 15 independent primary cultures.

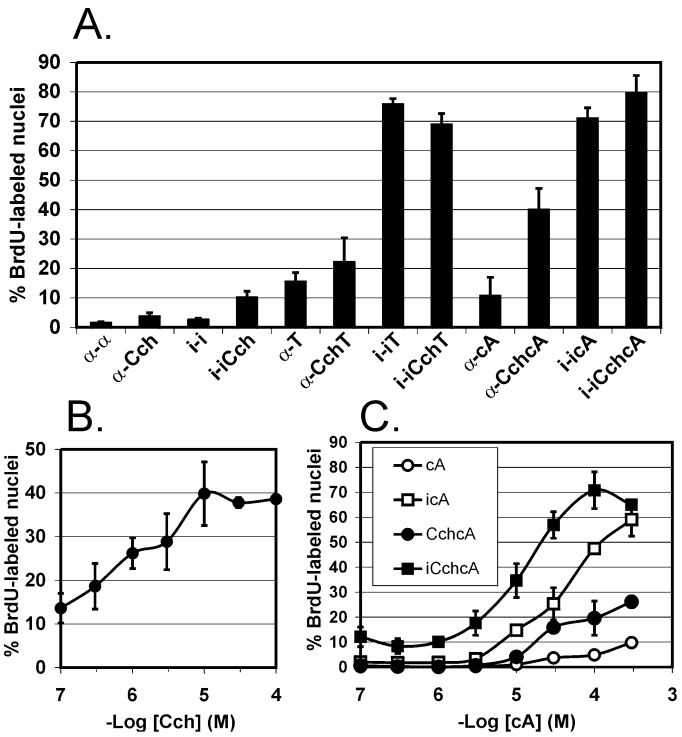

FIG. 1. A, Stimulation of DNA synthesis in dog thyrocytes cultured in the absence (α-) or in the presence of insulin (i-) for 4 days. Cells were then stimulated for 48 h with various combinations of insulin (i), carbachol (Cch), TSH (T), or $(Bu)_2$cAMP (cA). B and C, Cells cultured for 4 days in the absence of insulin were then stimulated for 48 h in the presence of $(Bu)_2$cAMP with different concentrations of carbachol (B), or with different concentrations of $(Bu)_2$cAMP (cA) in the presence of insulin (i), carbachol (Cch) or both (iCch) (C). BrdU was present during the last 24 h, and the fraction (mean + range of duplicate dishes) of nuclei having incorporated BrdU was determined.

The different results obtained with the combinations of carbachol and TSH or the nonhydrolysable $(Bu)_2$cAMP are likely to be explained by the fact that carbachol was previously described to activate Ca^{2+} calmodulin-dependent phosphodiesterases, which hydrolyze cAMP (18). The cellular cAMP concentration resulting from the opposite effects of TSH and carbachol could thus be insufficient to trigger DNA synthesis in a large number of cells. This explanation was not immediately apparent in our previous report (10), because the inhibitory effect of carbachol on the accumulation of cAMP was generally insufficient to counteract the mitogenesis triggered by maximal TSH concentrations in the presence of insulin (Fig. 1A, cond i-iCchT *vs.* i-iT), as reported previously (20). We reasoned that these apparently paradoxical results might indicate that lower cellular cAMP concentrations could suffice to elicit DNA synthesis in the combined presence of carbachol and insulin, compared with cells stimulated in the presence of insulin alone. Indeed, as shown on Fig. 1C, similar DNA synthesis stimulations were obtained using 5- to 10-fold lower concentrations of $(Bu)_2$cAMP in the presence of carbachol+insulin compared with cells stimulated in the presence of insulin alone. Figure 1C also illustrates that the addition of carbachol to the condition insulin plus $(Bu)_2$cAMP resulted in an increase of the fraction of labeled nuclei, suggesting that the two permissive factors can cooperate through additive effects.

The hypertrophic effect of carbachol is not sufficient to permit $(Bu)_2$cAMP-induced DNA synthesis

We have shown (27) that the muscarinic antagonist atropine completely abolishes all the effects of carbachol in dog thyrocytes, including Ca^{2+} mobilization within 10 sec and H_2O_2 generation (within 1 min) which depends on both Ca^{2+} and protein kinase C activation. Atropine also prevented the permissive effect of carbachol for $(Bu)_2$cAMP mitogenic action (Fig. 2, same proliferative response in the condition α-CchcA +At than in the condition α-cA). It thus enables us to design experiments which were impossible using insulin, which it is difficult to wash out. Indeed in our previous experiments (10), the effect of insulin on DNA synthesis stimulated by TSH only partly declined two days after its washing out.

An important function lacking in the comitogenic action of TSH and cAMP is the increase of overall protein synthesis leading to the regular doubling of cell mass required for repetitive cell division. This cell mass increase is provided by insulin (10, 28). As carbachol stimulates cell hypertrophy to the same extent (10), we tested here whether this hypertrophy could be sufficient to permit DNA synthesis triggered by the cAMP-dependent pathway. We induced a durable cell

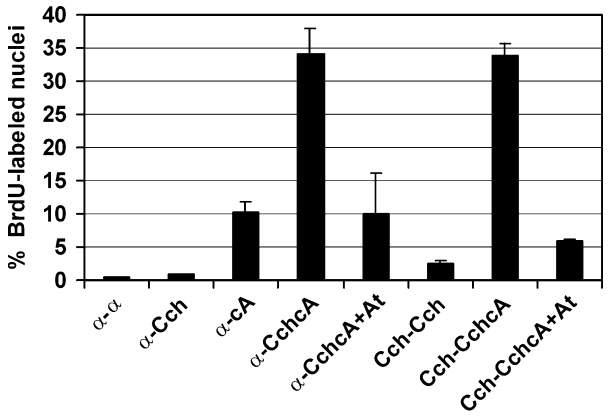

FIG. 2. Stimulation of DNA synthesis in dog thyrocytes pretreated (Cch-) or not (α-) with carbachol for 4 days to generate a hypertrophic response. Cells were then stimulated for 48 h in the absence or in the presence of carbachol (Cch) with $(Bu)_2$cAMP (cA) and atropine (At). BrdU was present during the last 24 h, and the fraction (mean + range of duplicate dishes) of nuclei having incorporated BrdU was determined.

hypertrophy by pretreating cells from day 1 with carbachol. At day 4, we stimulated cells with $(Bu)_2$cAMP in the presence of carbachol, and atropine was added to some dishes at this moment. As shown in Fig. 2, atropine completely inhibited the permissive effect of carbachol even after the hypertrophic carbachol pretreatment (Cch-CchcA+At *vs.* α-cA). Cell hypertrophy is thus not sufficient to permit the $(Bu)_2$cAMP-induced DNA synthesis, which required the simultaneous activity of the permissive factor. Interestingly, there was no desensitization of carbachol permissive action in these experiments (Cch-CchcA *vs.* α-CchcA) (Fig. 2).

Comitogenic effects of carbachol and $(Bu)_2$cAMP are integrated by cell cycle regulatory proteins

As shown in Fig. 3A, in quiescent cells cultured in the control medium for 4 days, the presence of cyclin A, cdc2 and the hyperphosphorylated form of pRb was very weak or undetectable. At 32 h, when most cells stimulated by insulin and $(Bu)_2$cAMP are in S phase, carbachol, and $(Bu)_2$cAMP alone had no detectable effects on the expression of cyclin A and cdc2 and on the hyperphosphorylation of pRb. $(Bu)_2$cAMP in the presence of carbachol greatly stimulated the accumulation of cyclin A and cdc2 and the hyperphosphorylation of Rb, as did $(Bu)_2$cAMP in the presence of insulin. In this experiment, insulin alone had a weak mitogenic effect (10% BrdU-labeled nuclei), which correlates with its weak effects on these three cell cycle regulators.

Carbachol like insulin overrides the repression of cyclins D by $(Bu)_2$cAMP

The hyperphosphorylation of pRb is generally considered to be initiated by the activation of cdk4/6 by cyclins D. After a stimulation of 20 h, a time corresponding for stimulated cells to a maximum of cells in mid to late G1 phase, carbachol increased the accumulation of cyclin D3, the most abundant cyclin D in quiescent dog thyrocytes, and of cyclins D2 and D1 (exposure times were longer), as did insulin (Fig. 3B). By contrast $(Bu)_2$cAMP alone paradoxically inhibited the accumulation of these three proteins, as we have already described it for TSH (6). As the positive effect of carbachol or insulin compensated for the negative effect of $(Bu)_2$cAMP, in all the experiments cyclin D3 levels were far higher in cells stimulated by $(Bu)_2$cAMP in the presence of carbachol or insulin than in cells treated with $(Bu)_2$cAMP alone. These effects were reproduced in 9 independent primary cultures. Figure 3C illustrates the additivity of the effects of carbachol and insulin on the accumulation of cyclin D3 in the presence of $(Bu)_2$cAMP.

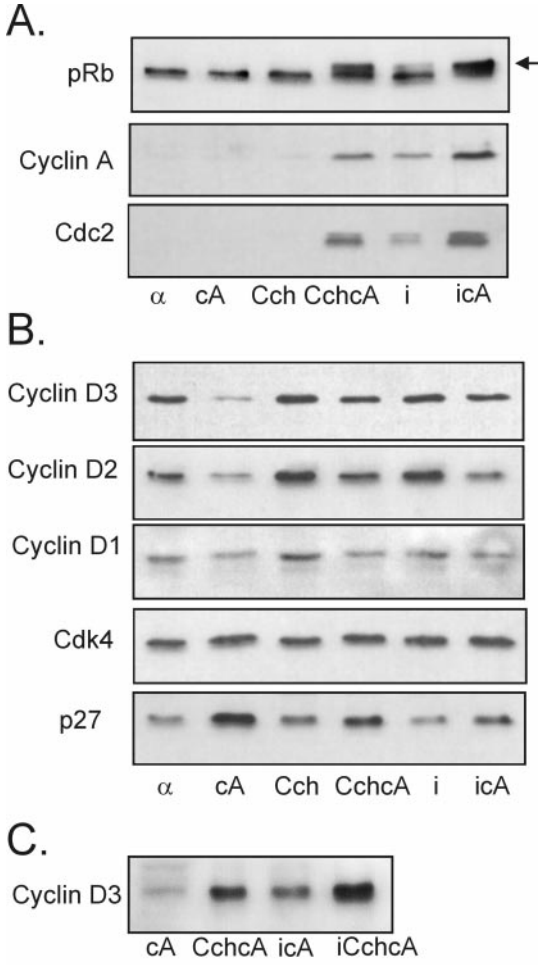

FIG. 3. Western blotting analysis of the phosphorylation of pRb, and the accumulation of various cell cycle regulatory proteins in dog thyrocytes stimulated at day 4 for 32 h (A) or 20 h (B and C) by $(Bu)_2$cAMP (cA), carbachol (Cch) or insulin (i). In (A) the position of the band corresponding to the hyperphosphorylated forms of pRb is indicated by an *arrow*.

$(Bu)_2$cAMP reproduced the positive effect of TSH (29, 30) on the accumulation of the cdk inhibitor $p27^{kip1}$. This positive effect was also observed in the presence of carbachol (Fig. 3B).

Requirement for cyclin D3 in cell cycle progression stimulated by $(Bu)_2$cAMP and carbachol

Because carbachol also stimulated the accumulation of cyclins D1 and D2 (Fig. 3B), the determining importance of

cyclin D3 was assessed by the microinjection of the previously characterized cyclin D3 neutralizing antibody DCS-29 (13, 24). This highly reactive and perfectly monospecific antibody does not recognize cyclins D1 and D2. It recognizes an epitope (aa 241–260) close to the cyclin box domain of interaction with cdk4 and precipitates cyclin D3 with very low associated kinase activity (24). As illustrated in Fig. 4, cells microinjected with DCS-29 and stimulated by $(Bu)_2$cAMP and carbachol were prevented from entering DNA synthesis (61.5+/−4.5% (mean +/− range from two different experiments) of inhibition of BrdU labeling in microinjected cells compared with neighboring nonmicroinjected cells). By contrast, the microinjection of a control IgG did not inhibit DNA synthesis (Fig. 4) as in many previous experiments (13). In the present experiments, the microinjection of DCS-29 also prevented the stimulation of DNA synthesis by $(Bu)_2$cAMP and insulin to the same extent (60% inhibition). The inhibition by DCS-29 of the cAMP-dependent entry into S phase supported by carbachol and insulin was very specific, because the microinjection of this antibody did not affect the stimulation of DNA synthesis in hepatocyte growth factor-treated dog thyrocytes that express higher levels of cyclins D1 and D2 in addition of cyclin D3, as previously shown (13).

Cyclin D3-cdk4 complexes integrate distinct $(Bu)_2$cAMP and carbachol effects

Quiescent dog thyrocytes expressed cdk4 before mitogenic stimulation (cond α, Fig. 3B). The abundance of cdk4 was unchanged (or very weakly increased in some experiments) 20 h after stimulation of the different agents (Figs. 3B and 5). Cyclin D3-cdk4 immune complexes were analyzed 20 h after cell stimulation by $(Bu)_2$cAMP and carbachol (Fig. 5). When cyclin D3 was immunoprecipitated using DCS-28, which recognizes the carboxy terminus of cyclin D3 (24), the same pattern of regulation of cyclin D3 accumulation was observed than by direct Western blotting analysis of the whole cell lysates (Fig. 3B), with a marked repression by $(Bu)_2$cAMP overridden by carbachol and insulin (Fig. 5, A and B). Despite high concentrations of cyclin D3 and cdk4 in cells cultured with carbachol alone, cdk4 was almost absent in cyclin D3 immunoprecipitates. In the presence of carbachol, as in the presence of insulin, the association of cdk4 with cyclin D3 was strongly stimulated by $(Bu)_2$cAMP, though cyclin D3 expression was slightly reduced (Fig. 5, A and B). By contrast, in cells stimulated by $(Bu)_2$cAMP alone, only a weak presence of cyclin D3-cdk4 complexes was detected [$(Bu)_2$cAMP alone had a weak mitogenic effect (9.2% BrdU-labeled nuclei)]. In this condition, the low amount of cyclin D3-cdk4 complexes was related to the low overall amount of cyclin D3, which thus appeared to be rate limiting for the assembly of complexes induced by $(Bu)_2$cAMP (Fig. 5, A and B).

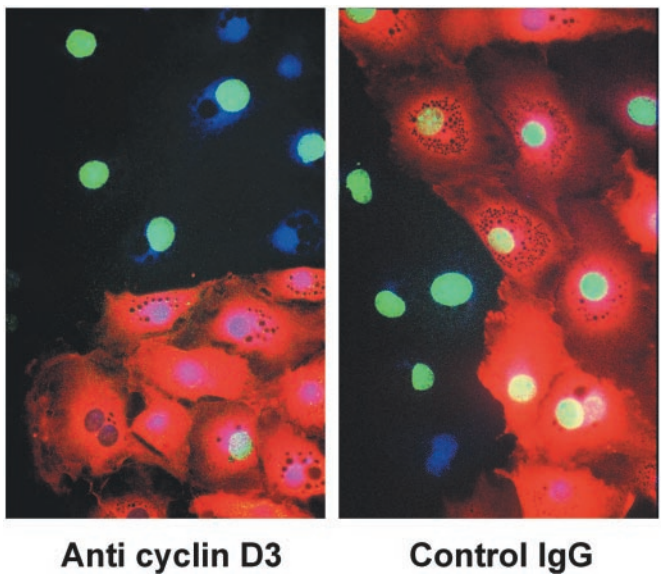

FIG. 4. Requirement of cyclin D3 for DNA synthesis stimulated by $(Bu)_2$cAMP in the presence of carbachol. The neutralizing cyclin D3 monoclonal antibody (DCS-29, 6 mg/ml) or the control IgG (6 mg/ml) were microinjected at day 4 just before the stimulation by $(Bu)_2$cAMP and carbachol for 48 h. BrdU was added for the last 24 h. Nuclei were identified by Hoechst 33342 staining of DNA (*blue fluorescence*). Microinjected cells were identified by the immunodetection of the injected antibody (*red fluorescence*). BrdU was coimmunodetected (*green fluorescence*). The experiment was repeated twice. In each experiment, a total of 600 cells were injected in each condition.

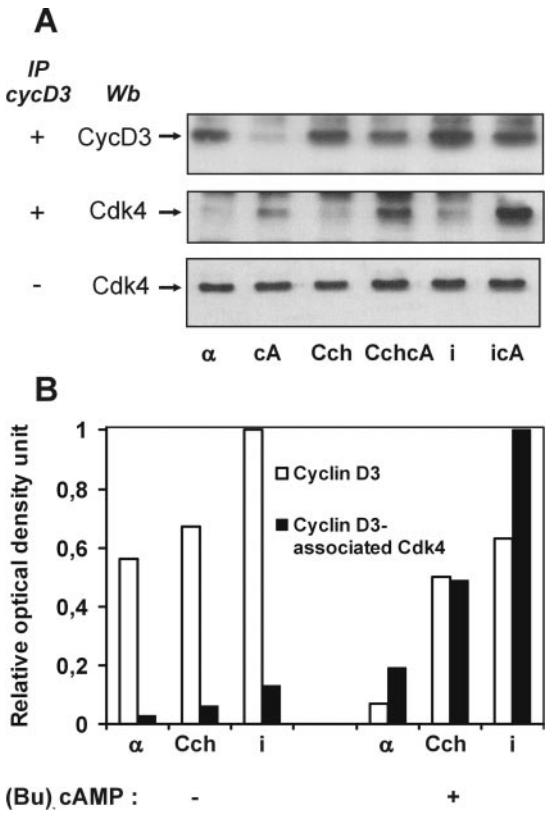

FIG. 5. Analysis of cyclin D3-cdk4 complexes in dog thyrocytes stimulated at day 4 for 20 h by $(Bu)_2$cAMP (cA) in the absence or in the presence of carbachol (Cch) or insulin (i). The presence of cyclin D3 and cdk4 was analyzed by Western blotting (wb) from complexes immunoprecipitated (IP) using a specific cyclin D3 antibody (DCS-28) or from the whole cell lysate (A). B, Laser scanning densitometry of the bands corresponding to cyclin D3 and cdk4 precipitated by the cyclin D3 antibody as shown in A.

The nuclear import of cdk4 depends on both $(Bu)_2$cAMP and carbachol

In quiescent cells maintained in control medium (condition α), cdk4 was distributed in both the cytoplasm and nucleus (Fig. 6). In response to the combination of $(Bu)_2$cAMP and carbachol, a majority of cells displayed a strong increase of the nuclear staining of cdk4 at the expense of the cytoplasmic labeling, suggesting a nuclear translocation of cdk4 (Fig. 6). This nuclear import was observed only in a very few cells stimulated by $(Bu)_2$cAMP or carbachol alone (Fig. 6). As previously shown in cells stimulated by TSH and insulin, $(Bu)_2$cAMP in the presence of carbachol also increased the nuclear detection of cyclin D3 by the DCS-22 monoclonal antibody [data not shown but similar to previous reports (6, 13)]. This effect reflects both the unmasking of the DCS-22 epitope (due to modifications of protein-protein interaction or conformational changes) and a nuclear translocation of cyclin D3, which parallels the nuclear import of cdk4 (13).

Late G1 phase progression depends on cAMP but no longer on carbachol

In dog thyrocytes cultivated in the presence of insulin, the stimulation of DNA synthesis by the general adenylyl cyclase activator forskolin requires its continuous presence until a very late G1 stage (31, 32). Even after the required induction of stable nuclear cyclin D3-cdk4 complexes, dog thyrocytes still depend on cAMP for pRb phosphorylation and commitment to DNA synthesis (31). This provided the evidence for an additional cAMP-dependent control of the passage through the restriction point (the no-return checkpoint in late G1 phase where further progression toward S phase and cell cycle completion becomes independent of external factors). Whether such a crucial control depends on permissive factors was ignored. The experiment illustrated in Fig. 7 was designed to assess whether the last decisional control in G1 is exerted by cAMP, carbachol used as a surrogate fully mimicking insulin action on cell cycle regulatory proteins, or both comitogens together.

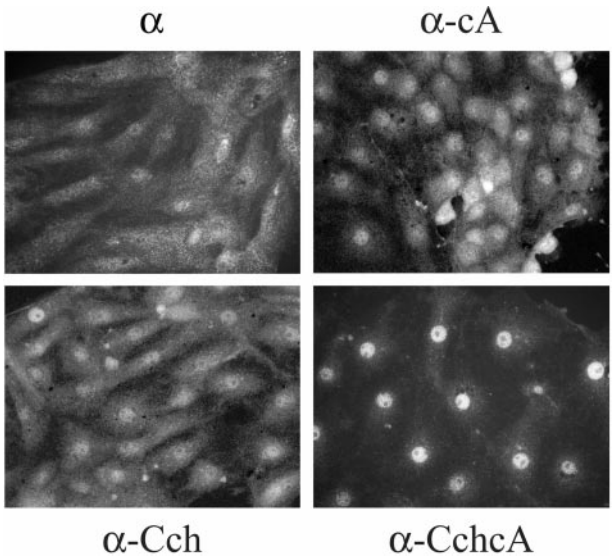

FIG. 6. Immunofluorescence labeling of cdk4 showing the increase of the nuclear staining of cdk4 at the expense of its cytoplasmic labeling in cells stimulated for 20 h by $(Bu)_2$cAMP in the presence of carbachol (CchcA) but not in cells stimulated by $(Bu)_2$cAMP (cA) or carbachol (Cch) alone. α, Nonstimulated control cells.

Cells were stimulated during 16 h or 20 h with carbachol and $(Bu)_2$cAMP. At this time, either $(Bu)_2$cAMP was removed, or atropine was added to deactivate the signaling cascades of carbachol, or the cells were maintained in the medium carbachol+$(Bu)_2$cAMP. As mentioned above, atropine immediately and completely blocks the mobilization of Ca^{2+} and the Ca^{2+} and PKC-dependent generation of H_2O_2 induced by carbachol in dog thyrocytes (27). In the present experiment, it also completely reversed the activatory phosphorylation of p42/p44 MAP kinases by carbachol (Fig. 7B). As shown in Fig. 7A, cells deprived of $(Bu)_2$cAMP were largely prevented from entering S phase, already 2 h after cell rinsing, confirming in the presence of carbachol the continuous requirement for cAMP until a late G1 commitment/restriction point (31). On the contrary, the neutralization of carbachol by addition of atropine did not affect the progressive entry of cells into S phase, at least for 6–8 h (Fig. 7A). These data show that the late stages of G1 phase still depend on cAMP but no longer on the continuous activity of carbachol. This suggests that the entry into S phase can be regulated by cAMP alone.

As cyclin D3 is crucial for the permissive effect of carbachol, we investigated the fate of cyclin D3 after atropine addition to cells stimulated for 20 h with $(Bu)_2$cAMP and carbachol. Four hours after atropine addition, cyclin D3 amount was at the same level than in the condition $(Bu)_2$cAMP+carbachol without atropine, and much higher than with $(Bu)_2$cAMP alone (cA) (Fig. 7B). This amount remained unchanged 8 h after atropine addition, whereas it continued to increase in the presence of $(Bu)_2$cAMP+carbachol without atropine. The amount of cyclin D3 thus remained stable after carbachol neutralization by atropine addition, at least for 8 h. This was not due to a stabilization of cyclin D3 because, as studied after a cycloheximide treatment in control cells, carbachol or carbachol+$(Bu)_2$cAMP stimulated cells, it had a half life of less than 2 h (Fig. 7C). The continuous presence of cyclin D3 after atropine administration at 20 h thus implies that at this stage the synthesis of cyclin D3 had become independent of a sustained activation of carbachol signaling cascades.

Discussion

In various *in vitro* thyroid cell systems, including primary cultures of canine and human thyrocytes, rat thyroid cell lines, as probably in adult human thyroid gland *in vivo*, the stimulation of cell proliferation associated with goitre formation by TSH through cAMP depends on the cooperation of other comitogenic factors, generally the insulin-like growth factors and insulin (2–4, 26, 33). In this work, we show that carbachol, through muscarinic receptors coupled to phospholipase C, can replace insulin as a permissive factor for the cAMP-dependent mitogenesis in dog thyrocytes. The existence of a cholinergic control of thyroid activity has long been suspected (34). Thyroid follicles receive parasympathetic innervation from cholinergic neurons (14, 35), and in rats the localized section of these nerves has been reported

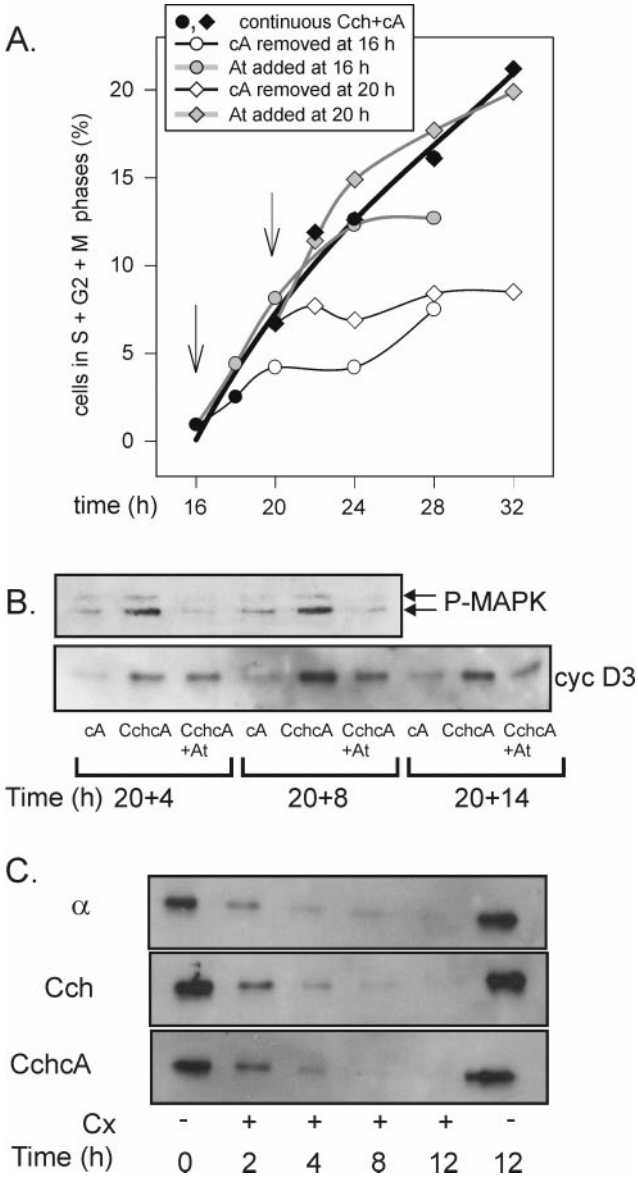

FIG. 7. A, Kinetics of S phase entry of dog thyrocytes after stimulation with $(Bu)_2$cAMP and carbachol, and then either removal of $(Bu)_2$cAMP or neutralization of carbachol by atropine. Thyrocytes were stimulated at 0 h with $(Bu)_2$cAMP (cA) and carbachol (Cch). At 16 h (●, ○, ○) or 20 h (◆, ◇, ◇) (*arrows*), cells were either rapidly rinsed and immediately replaced in the culture medium with carbachol alone to deprive cells of $(Bu)_2$cAMP (○, ◇); or atropine was added to antagonise the activity of carbachol (○, ◇) or cells were maintained in the Cch+cA medium (●, ◆). At the indicated times, cells were then fixed and the percentage of cells in S, G2, and M phases were determined from the patterns of PCNA immunofluorescent labeling. B, Kinetics of cyclin D3 accumulation and p42/p44 MAP kinase phosphorylation after atropine addition to dog thyrocytes stimulated by carbachol and $(Bu)_2$cAMP. Thyrocytes were stimulated for 20 h with $(Bu)_2$cAMP (cA) or carbachol+$(Bu)_2$cAMP (CchcA). At 20 h, some carbachol+$(Bu)_2$cAMP-treated cells received atropine (At), or medium remained unchanged, and cyclin D3 accumulation and the presence of p42 and p44 phosphoMAPkinases (PMAPK, *arrows*) were analyzed by Western blotting 4 h, 8 h, or 14 h afterward. **C,** Kinetics of cyclin D3 disappearance after cycloheximide addition. Dog thyrocytes were stimulated or not (α) during 20 h with carbachol or carbachol+$(Bu)_2$cAMP. Cycloheximide was added (+) or not (−) at this time, and cyclin D3 accumulation was analyzed by Western blotting 0 h, 2 h, 4 h, 8 h, or 12 h afterward.

to significantly impair the methylmercaptoimidazole-induced goitrogenic response and the compensatory growth after hemithyroidectomy (36). Interestingly, concomitant hyperactivations of adenylyl cyclase and phospholipase C in thyroid of transgenic mice result in growth stimulation and malignant transformation (37). Nevertheless the physiological relevance of our present finding is not immediately apparent, because the comitogenic activity of carbachol could be demonstrated when using the nonhydrolysable cAMP analog $(Bu)_2$cAMP but hardly in the presence of the physiological stimulus TSH. This divergent result can be easily understood because carbachol activates a Ca^{2+}calmodulin-dependent cAMP-phosphodiesterase and thus diminishes the cellular level of cAMP raised by TSH (18). The apparent paradox that carbachol only weakly affected DNA synthesis triggered by TSH+insulin is explained here by the observation that when both permissive agents were present, a lower cAMP concentration sufficed to induce a comparable proliferative response than in the presence of insulin alone.

The additivity of comitogenic effects of carbachol and insulin in the presence of $(Bu)_2$cAMP is consistent with their partly different signaling cascades. Carbachol strongly activates a phospholipase C/Ca^{2+}, diacylglycerol pathway leading to a potent activation of MAP kinases (7, 19, 27), but it weakly activates the PI3 kinase/PKB pathway (Vandeput, F., and K. Coulonval, unpublished data). By contrast insulin strongly activates the PI3 kinase/PKB pathway (9) but weakly the Ras/MAP kinases pathway (8, 26). Nevertheless, the initially distinct signaling pathways of carbachol and insulin converge on two essential functions that are lacking in the mitogenic action of TSH and cAMP, the stimulation of cell hypertrophy (10), and as shown here, the accumulation of cyclin D3, which is uniquely positioned to play a critical role in the cell cycle commitment. This allows us to confirm and generalize our model of the necessary functions that should be contributed by permissive factors to support the cAMP-dependent cell cycle (6). Furthermore, the present demonstration of convergent mechanisms of insulin and carbachol has suggested us to use carbachol and the immediate arrest of its activity by atropine as a valuable surrogate of insulin to precise the respective roles of comitogenic permissive factors and cAMP during cell cycle progression.

An important feature of the complementarity of comitogenic effects of insulin and TSH (cAMP) is the fact that the stimulation of the overall protein synthesis and accumulation required for a sustained cell proliferation is provided by insulin but not by TSH (10). The stimulation of protein synthesis by insulin and IGF-1 was thus envisaged as a prerequisite for the mitogenic effect of TSH (28). Here we have demonstrated that, in cells previously enlarged by a carbachol pretreatment, DNA synthesis did not respond to cAMP in the absence of a simultaneous activation of muscarinic receptors (*i.e.* in the presence of atropine; Fig. 2). This definitively confirms that cell hypertrophy, whereas it may be necessary, is not sufficient to permit the cAMP-dependent mitogenesis.

Like the stimulation of DNA synthesis, the accumulation of cyclin A and cdc2 and the hyperphosphorylation of pRb resulted from the synergistic interaction of carbachol and cAMP. In other models, the transcription of cyclin A and cdc2

genes depends on E2F transcription factors released by the hyperphosphorylation of pRb (38, 39). It thus reflects the cell cycle progression beyond the pRb checkpoint. The phosphorylations of pRb associated with the cell's commitment toward DNA replication and cell cycle completion are initiated by cdk4/6 activated by cyclins D (39–41). The neutralization of cyclin D3, the most abundant cyclin D in dog thyrocytes (6, 13), by microinjection of a blocking antibody prevented S phase entry triggered by the $(Bu)_2$cAMP in the presence of carbachol. However, while $(Bu)_2$cAMP mimicking TSH induced the formation of complexes of cyclin D3 and cdk4 and the nuclear translocation of these proteins, it paradoxically inhibited the basal accumulation of cyclin D3. Like insulin, carbachol overcame this inhibition, thus supplying cyclin D3, which appeared to be rate limiting for the assembly of cyclin D3-cdk4 complexes elicited by cAMP. Consistently, the effects of carbachol and insulin were additive on both cyclin D3 accumulation and DNA synthesis in the presence of $(Bu)_2$cAMP. Exactly as shown previously for the mitogenic stimulation by TSH and insulin (6), we thus demonstrate here that the formation and nuclear translocation of required cyclin D3-cdk4 complexes integrate the distinct but complementary effects of $(Bu)_2$cAMP and carbachol.

The mechanisms of cAMP-dependent cyclin D3-cdk4 assembly and nuclear import remain unclear. They have provided a unique example of such regulations as critical targets for cell cycle activation by a physiological stimulus. Recently cdk inhibitors including $p27^{kip1}$ have been suggested to be essential for both assembly and nuclear localization of cyclin D-cdk complexes (42, 43). In dog thyrocytes, the increase of $p27^{kip1}$ expression in response to TSH and $(Bu)_2$cAMP, which was independent of insulin and carbachol (Fig. 2B), might indeed provide the nuclear anchor for these complexes, but does not appear to be essential for their assembly (30).

In dog thyrocytes in the presence of insulin, the removal of the cAMP stimulus arrests with little (less than 2 h) delay the accumulation of phosphorylated pRb and the entry of cells into S phase, but does not disrupt nuclear cyclin D3-cdk4 complexes. This has implied that even after induction of stable nuclear cyclin D3-cdk4 complexes, dog thyrocytes still depend on cAMP for pRb phosphorylation and commitment to DNA synthesis (31). Here we have shown that, contrary to the interruption of cAMP stimulus, the neutralization of carbachol by atropine after a stimulation of 16 h or 20 h still permitted the entry of cells in S phase for at least 6–8 h. The requirement for the activity of the carbachol pathway during G1 phase is thus shorter than the requirement for the cAMP pathway. Therefore, whereas the progression during a first part of G1 depends on the synergy of both carbachol and cAMP, cAMP can control alone the transit through the late G1 restriction point. The G1 phase progression stimulated by cAMP thus appears to consist of at least two essential actions that are clearly dissociated: in a first stage, depending on the supportive presence of an agent that stimulates the accumulation of cyclin D3, cAMP induces the assembly and nuclear translocation of stable cyclin D3-cdk4 complexes, and then cAMP alone exerts an additional function which is rate limiting for the phosphorylation of pRb and the initiation of DNA synthesis.

Interestingly, the interruption of carbachol signaling by the addition of atropine arrested the further increase of cyclin D3 accumulation but did not lead to the disappearance of cyclin D3 during at least 8 h. This result is consistent with the hypothesis that once carbachol has accomplished its role of supplier of cyclin D3, its presence becomes dispensable for cell progression in late G1 and commitment to DNA synthesis controlled by cAMP alone. Nevertheless, this result was unexpected because, as confirmed here in control cells, carbachol or carbachol+$(Bu)_2$cAMP-treated cells, cyclin D3 is a labile protein that depends on sustained protein synthesis. Its persistent presence after arrest of carbachol stimulation contrasts with the general observation that the synthesis of other cyclins D strictly depends on sustained activation of mitogenic signaling cascades, which has led to their proposed role as growth factor sensor (44, 45). We can only speculate about the mechanism maintaining cyclin D3 synthesis. An E2F-dependent transcription of cyclin D3 gene (46) could have relayed the external stimulation during G1-phase progression. Such a mechanism might also explain the late increase of cyclin D3 accumulation that we previously noticed during the cell cycle progression of dog thyrocytes stimulated not only by growth factors but also by cAMP (in the presence of insulin) (13) and inhibited by transforming growth factor β (30).

To conclude, this study illustrates that the roles of the cAMP pathway and the permissive pathways in the regulation of dog thyrocyte proliferation are clearly different and complementary at the biochemical level as well as at the biological level. Their separate actions on cyclin D3 accumulation and activity are integrated by the formation and nuclear translocation of cyclin D3-cdk4 complexes that associate with $p27^{kip1}$ (30), allowing the passage of a first checkpoint before the last decisional restriction point controlled by cAMP alone where the cell is definitively committed to replicate its genome.

Though our results from a thyroid cell in primary culture do not exclude additional interactions at earlier steps of the signaling cascades of comitogens (26), they sharply contrast with the converse situation reported in immortal rat thyroid cell lines. In FRTL-5 cells, TSH and cAMP exert a priming effect which makes the cell more competent to progress into G1 phase in response to insulin/IGF-1, which can control alone the entry into S phase (4, 47–49). Further TSH presence is dispensable during G1 phase progression (49) and maintenance of high cAMP levels even delays DNA synthesis initiation (50). In these cells, TSH (cAMP) and insulin/IGF-1 actions seem to be integrated through their additive effects on cyclin D1 induction and $p27^{kip1}$ down-regulation (49, 51, 52). The molecular logic of the synergistic cell cycle regulation is therefore different in rat thyroid cell lines and dog thyroid primary culture.

Acknowledgments

We thank J. Gannon and T. Hunt for the cyclin A antibody.

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