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Breast cancer cell response to calcitonin: Modulation by growth-regulating agents

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Abstract

Calcitonin may induce cyclic AMP production by breast cancer cells and inhibit their growth. The molecular complex leading to cyclic AMP production in response to calcitonin is made of the calcitonin receptor coupled to the adenylate cyclase by at least one guanine nucleotide-binding protein (G-protein, of the G_s type). Our aim was to determine whether and how the responses of cells to calcitonin were modulated by growth-regulating agents not directly acting through the cyclic AMP pathway. We found that the cyclic AMP response to calcitonin was reduced after preincubation of cells with the mitogens 17β-estradiol and epidermal growth factor (EGF), while it was enhanced after preincubation with the growth inhibitors tamoxifen and $1,25(OH)_2D_3$, as well as with an antisense oligonucleotide to the proto-oncogene c-*myc*. Scatchard-plots revealed no significant change in the calcitonin receptor number or affinity. On the other hand, the cyclic AMP production of cells in response to activators unrelated to calcitonin, such as forskolin, a direct adenylate cyclase effector, and isoproterenol, a β-adrenergic receptor agonist, was modulated only weakly or not at all by the growth-regulating agents. This suggested that the effects observed were essentially calcitonin-specific and associated with events located between the calcitonin receptor and the adenylate cyclase. Since a G_o- or G_i-protein has been previously implicated in the calcitonin signal transduction, we tested the action of pertussis toxin, a specific inhibitor of these G-proteins. Pertussis toxin produced a general increase in the cyclic AMP response of cells to calcitonin; moreover, the toxin almost abolished the effect of mitogens and antimitogens on that parameter. We conclude that in breast cancer cells, the calcitonin receptor and the adenylate cyclase are coupled by at least one G_o/G_i-protein sensitive to growth-regulating agents; this results in a modulation of the cyclic AMP response to calcitonin by these agents. On the other hand, the growth-inhibitory effect of calcitonin on breast cancer cells was reduced by 17β-estradiol and enhanced by tamoxifen. We suggest that this could be a consequence of changes in cyclic AMP levels and deserves further investigation.

Keywords: Calcitonin; Estrogen; Tamoxifen; cAMP; Cell growth; Breast cancer

1. Introduction

Calcitonin is a polypeptidic hormone that inhibits bone resorption and lowers serum Ca^{2+} by interacting with specific receptors on bone and kidney cells. This property has led to the use of pharmacological doses of calcitonin in the treatment of conditions characterized by an increase in osteoclast activity, such as cancer-associated hypercalcemia or osteoporosis (Azria, 1989). On the other hand, calcitonin receptors are present on several breast cancer cell lines in which the hormone triggers cyclic AMP production (Findlay et al., 1980). Calcitonin activates a cyclic AMP-dependent protein kinase (protein kinase A); forskolin and 8-bromo-cyclic AMP have similar activity. These drugs may inhibit breast cancer cell growth, maybe in part by interfering with the protein kinase C-mediated induction of the proto-oncogene c-*jun* by mitogens (Lacroix and Body, 1997). This inhibitory action of calcitonin on breast cancer cell growth could be clinically relevant but has been very little studied so far.

The mechanisms leading to cyclic AMP production and its modulation of cell growth have been widely studied. In contrast, much less is known on how these mechanisms are modulated by drugs essentially acting through other signalling pathways. For instance, it has been shown that 17β-estradiol, whose primary target is the estrogen receptor, inhibits the parathyroid hormone-induced cyclic AMP production in osteoblast-like cells, in part by acting on the adenylate cyclase and/or on the guanine nucleotide regu-

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latory protein (G-protein)-adenylate cyclase complex (Monroe and Tashjian, 1996). It also appears that epidermal growth factor (EGF), an activator of the phospholipase C-protein kinase C pathway, may inhibit the secretagogue-induced cyclic AMP production in pancreatic acini by acting on pertussis toxin-sensitive G-proteins (Stryjek-Kaminska et al., 1996). In the present work, we have been searching for similar modulations in breast cancer cells and, considering the possibility that these cells might respond in vivo to pharmacological calcitonin concentrations, we have chosen this hormone as the cyclic AMP inducer. Due to their clinical interest as cell-cycle modulators, 17β-estradiol, EGF, tamoxifen and 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) were used as breast cancer cells effectors not directly associated with the cyclic AMP pathway (Eisman, 1984; Carpenter and Cohen, 1990).

Fig. 1 summarizes the role of the various molecules involved in this report and should help to understand the results.

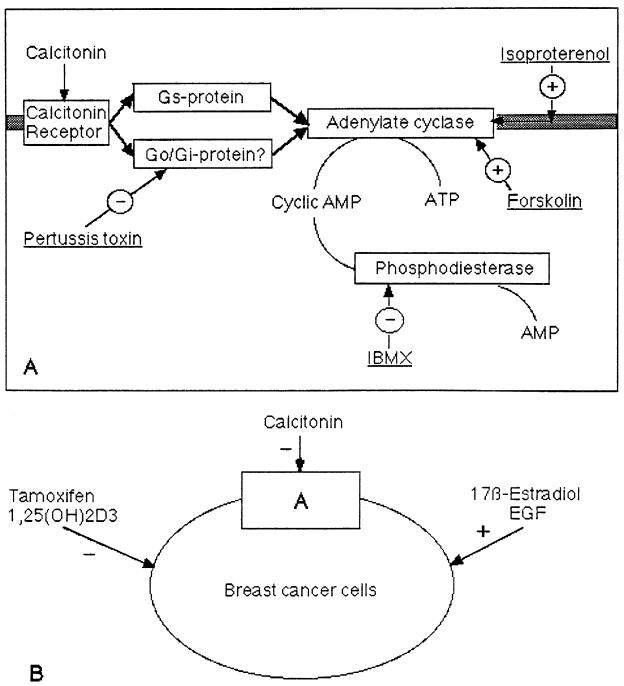

Fig. 1. Diagram representing the molecules involved in the study. (A) Calcitonin stimulates cyclic AMP production through a molecular cascade composed successively of the calcitonin receptor, one (a $G_{stimulatory}$ or G_s) or more guanine nucleotide-binding protein(s)(G-protein(s)) and the adenylate cyclase. We suggest here that, besides the G_s-protein, a pertussis toxin-sensitive G_{other} or G_o /$G_{inhibitory}$ or G_i-protein belongs to this cascade. The adenylate cyclase can also be directly stimulated by forskolin and indirectly by isoproterenol acting through the β-adrenergic receptor (not shown here). Cyclic AMP is synthesized by the adenylate cyclase from the precursor ATP; it is converted into the inactive AMP by a phosphodiesterase, an event prevented by IBMX. (B) Calcitonin (and cyclic AMP) is a negative regulator of breast cancer cell proliferation, as are tamoxifen and $1,25(OH)_2D_3$; in contrast, 17β-estradiol and EGF are positive regulators of proliferation.

We show that in the three cell lines that we used, the cyclic AMP production in response to calcitonin was decreased by the two mitogens (17β-estradiol and EGF) and increased by the two antimitogens (tamoxifen and $1,25(OH)_2D_3$), by a mechanism involving at least in part a pertussis toxin-sensitive protein associated with the calcitonin receptor. Moreover, we show that the cell growth inhibitory action of calcitonin on T-47D cells was enhanced by tamoxifen and lowered by 17β-estradiol.

2. Materials and methods

2.1. Cells

T-47D and MCF (Michigan Cancer Foundation)-7 breast cancer cell lines were from ATCC. IBEP (Institut Bordet Epanchement Pleural)-2 is a new cell line obtained in our laboratory. IBEP-2 cells originated from a pleural effusion in a woman with breast carcinoma and bone metastases. Like T-47D and MCF-7, they were estradiol receptor-positive (manuscript submitted) and possessed calcitonin receptors (12 500 sites/cell, $K_d = 0.6$ nM). IBEP-2 cells were not used at passages (1:5 splitting) higher than 5. The three cell lines were shown to respond to calcitonin by an increase in cyclic AMP production.

2.2. Reagents

Salmon calcitonin was from Saxon Biochemicals (Hannover, Germany). From Sigma (St. Louis, MO), we obtained: 17β-estradiol; EGF; forskolin, a drug interacting directly with- and activating the cAMP-producing enzyme adenylate cyclase (Seamon and Daly, 1981); isoproterenol, which activates indirectly the adenylate cyclase through the β-adrenergic receptors (Marchetti et al., 1990); 3-isobutyl-1-methylxanthine (IBMX), an inhibitor of the cyclic AMP-degrading enzyme phosphodiesterase; pertussis toxin, which inhibits the activity of G_i/G_o proteins by altering a cysteine in their alpha subunit (Gilman, 1987); 4′,6-diamidino-2-phenylindole, a fluorescent dye specific for DNA (Sorger and Germinario, 1983). Tamoxifen was from ICI Biochemicals (Macclesfield, UK), $1,25(OH)_2D_3$ was given by Roche (Basle, Switzerland). The antisense to c-*myc* (‘anti-Myc’) was a phosphorothioate oligonucleotide 5′-AACGTTGAGGGGCAT-3′, a sequence corresponding to the translation-initiation region of exon 2 of the *c-myc* gene (Chien et al., 1994). As a control, we used a ‘nonsense’ 5′-AGGGCAGTTAGACTG-3′ sequence containing the same nucleotides as the antisense, but in a random distribution (‘non-Myc’). These oligonucleotides were from Eurogentec (Seraing, Belgium). Iodinated salmon calcitonin (approximately 2000 Ci/mmol) was from Amersham, UK.

2.3. Cell culture

Cells were cultured in phenol red-free RPMI 1640 medium (GIBCO, Grand Island, NY), supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), HEPES (10 mM) and 5% fetal calf serum. Cells were incubated in a humidified atmosphere of 95% air–5% CO_2 at 37°C. For cyclic AMP experiments, breast cancer cells were seeded in 12- or 24-wells plates (Nunclon, Nunc) at a density of 30 000 or 15 000 cells/well in phenol red-free medium containing 5% fetal calf serum charcoal-treated to remove its endogenous steroids (Lippman et al., 1976). At preconfluence, cells were treated with 17β-estradiol, EGF, tamoxifen and 1,25(OH)$_2$D$_3$ ± calcitonin for 48 or 96 h (proliferation studies) in the same medium excepted that only 2% charcoal-treated fetal calf serum was used.

2.4. Cyclic AMP determination

Cells were preincubated for 20 min in fetal calf serum-free RPMI-1640, in presence or not of IBMX (2 mM). When used, pertussis toxin was also added during that preincubation period. Calcitonin, isoproterenol and forskolin were added for a further 20-min incubation, after which the cells were washed three times with RPMI-1640 and fixed in 95% isopropanol/5% H_2O for 1 h at 4°C. Cyclic AMP was dried by evaporation and resuspended in 0.05 M sodium acetate (pH 5.2), and measured using an in-house radioimmunoassay (range of sensitivity: between 0.0025 and 25 pmol/100 μl) as previously described (Lacroix et al., 1996).

2.5. Binding studies

Calcitonin binding studies were adapted from methods previously described (Body et al., 1990). Cells contained in 24-wells plates were washed twice and incubated in RPMI-1640 medium at 37°C for 1 h, with the appropriate amounts of labelled calcitonin. An excess ($5 \cdot 10^{-7}$ M) of cold salmon calcitonin was added to some wells to saturate the calcitonin receptors and estimate the non-specific binding. After incubation, cells were washed twice with ice-cold PBS and lysed in 0.5 M NaOH. Radioactivity was measured in a Crystal Gamma System (Packard). Specific binding of labelled calcitonin was calculated by subtracting the values of bound radioactivity obtained in absence and in presence of the cold hormone.

2.6. Cell proliferation studies

DNA determinations were made as previously described (Lacroix et al., 1996). Briefly, trypsinised cells were washed 2 times and sonicated in 10 mM tris–HCl, 1 mM $MgCl_2$ (pH 7.4). DNA concentrations were then measured in sonicated samples by fluorimetry, using 4′,6-diamidino-2-phenylindole (Sorger and Germinario, 1983) as intercalating agent.

In some cases, breast cancer cells were counted after trypsinization.

2.7. Statistics

All values are expressed as mean ± S.D or S.E. Statistical significance of differences in mean values was assessed by two-tailed Student's *t*-test. A P-value < 0.05 was considered significant.

3. Results

3.1. Modulation of cyclic AMP response of breast cancer cells to calcitonin by cell proliferation-regulating agents

We verified that calcitonin induced cyclic AMP production in IBEP-2, MCF-7 and T-47D cell lines (Findlay et al., 1980). We found that the cyclic AMP response of all three cell lines to 10^{-8} M calcitonin was lowered after a 48 h incubation with 10^{-8} M 17β-estradiol or 10 ng/ml EGF. In contrast, breast cancer cells incubated for the same time with tamoxifen (10^{-6} M) or with 1,25(OH)$_2$D$_3$ (10^{-8} M) showed an increased cyclic AMP response to calcitonin. For example, in IBEP-2 cells, control values were 10.1 ± 0.6 pmol cyclic AMP/μg DNA. They decreased to 5.9 ± 0.8 and 5.5 ± 0.9 pmol cyclic AMP/μg

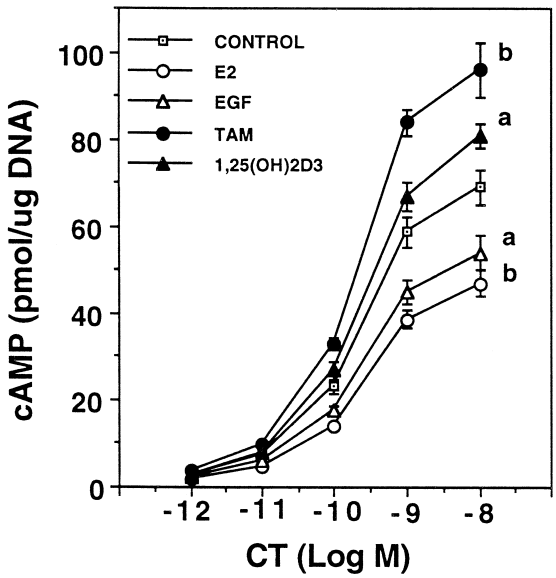

Fig. 2. Dose-dependent cyclic AMP response of T-47D breast cancer cells to calcitonin: effects of a preincubation of cells with various growth regulators (10^{-8} M 17β-estradiol; 10 ng/ml EGF; 10^{-6} M tamoxifen; 10^{-8} M 1,25(OH)$_2$D$_3$). Results are mean ± S.D. ($n = 3$). For clarity purposes, significance levels are indicated only for the highest calcitonin concentration (10^{-8} M). (a) $P < 0.05$; (b) $P < 0.01$. The modulation was, however, significant at all concentrations tested (at least $P < 0.05$), except for 1,25(OH)$_2$D$_3$ at 10^{-12} to 10^{-10} M calcitonin ($P < 0.1$).

DNA ($n = 3$, $P < 0.05$ versus control) after pretreatment with 17β-estradiol or EGF, respectively, whereas they increased to 13.6 ± 0.9 and 14.0 ± 1.2 pmol cyclic AMP/μg DNA ($n = 3$, $P < 0.05$ versus control) after pretreatment with tamoxifen or 1,25(OH)$_2$D$_3$. Similar results were obtained with MCF-7 (data not shown) and T-47D breast cancer cells lines (Fig. 2). The above-mentioned effects were significant (at least $P < 0.05$) at all calcitonin concentrations that were tested (10^{-12} to 10^{-8} M), except for 1,25(OH)$_2$D$_3$ which significantly increased the response to calcitonin only at 10^{-9} or 10^{-8} M. These effects were observed independently of the presence of IBMX, an inhibitor of the cyclic AMP-degrading enzyme phosphodiesterase (data not shown), suggesting that they were not due to changes in cyclic AMP degradation rates.

3.2. Specificity of the modulation of breast cancer cells response to calcitonin

Fig. 3 shows that in T-47D cells: (a) the growth-regulating agents modulated DNA synthesis and cyclic AMP production in response to calcitonin in an opposite way and (b) the cyclic AMP production in response to the adenylate cyclase activators forskolin and isoproterenol was modulated only weakly or not at all, with the exception of the response to isoproterenol after incubation of cells with 17β-estradiol, an effect probably due to an increase in beta-adrenergic receptor number (Marchetti et al., 1990). For forskolin, only the cyclic AMP values obtained after incubation of T-47D with 17β-estradiol or tamoxifen were reproducibly different from control values. Similar observations were made with MCF-7 and IBEP-2 cell lines (data not shown).

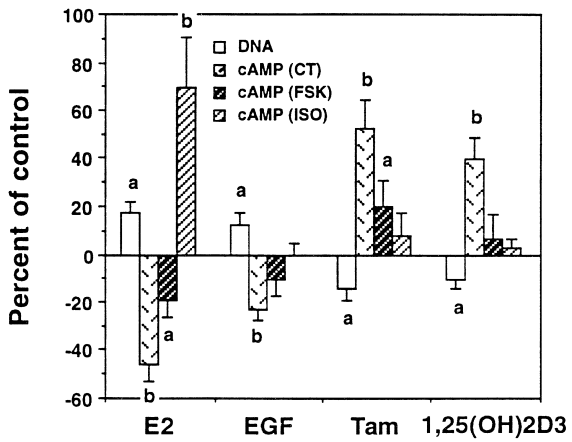

Fig. 3. DNA concentrations and cyclic AMP response to 10^{-8} M calcitonin, 10^{-6} M forskolin (FSK) and 10^{-5} M isoproterenol (ISO) in T-47D breast cancer cells: effects of a preincubation of cells with various growth regulators (concentrations as in Fig. 1). Control = 0. Results are mean $\pm$ S.D. ($n = 4$); (a) $P < 0.05$ and (b) $P < 0.01$ compared with control.

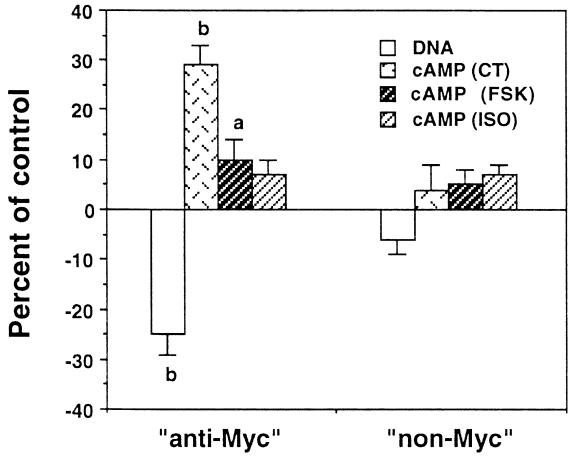

Fig. 4. DNA concentrations and cyclic AMP response to 10^{-8} M calcitonin, 10^{-6} M forskolin and 10^{-5} M isoproterenol in T-47D breast cancer cells: effect of a preincubation of cells with an anti-c-Myc ('anti-Myc') and a non-c-Myc ('non-Myc') oligonucleotide (10^{-6} M each). Results are mean $\pm$ S.D. ($n = 4$); (a) $P < 0.05$; (b) $P < 0.01$.

3.3. Effects of an anti-c-myc antisense oligonucleotide

In breast cancer cells, an increase in the proto-oncogene c-*myc* mRNA and protein levels is observed in response to mitogens, while anti-estrogens have an opposite effect (Musgrove et al., 1993). More generally, anti-c-*myc* antisense oligonucleotides reduce cell growth by preventing the cell-cycle transition in G_1 (Evan and Littlewood, 1993; Chien et al., 1994).

We tested if the inhibition of c-*myc* expression by an antisense oligonucleotide was sufficient to up-regulate the cyclic AMP response of breast cancer cells to calcitonin. As shown in Fig. 4, after incubation with the 'anti-Myc' alone (10^{-6} M), DNA concentrations were reduced and the response of T-47D cells to calcitonin, isoproterenol and forskolin was regulated exactly in the same way as observed with 1,25(OH)$_2$D$_3$ and tamoxifen (compare to Fig. 3). The control 'non-Myc' exerted no significant effect. Furthermore, the anti-c-*myc* antisense was also able to

Table 1
Combined effects of an anti-c-Myc oligonucleotide and mitogens on DNA levels and cyclic AMP response to calcitonin in breast cancer cells

	DNA (μg/well)	cAMP (pmol/μg DNA)
Control	4.7 ± 0.3	52.7 ± 3.7
Antimyc	3.7 ± 0.3[a]	73.1 ± 4.1[a]
E2	6.1 ± 0.5[a]	34.0 ± 2.3[a]
E2 + antimyc	5.1 ± 0.4	47.3 ± 2.6
EGF	5.7 ± 0.2[a]	43.1 ± 3.4[a]
EGF + antimyc	4.5 ± 0.3	50.9 ± 2.1

Cells were incubated for 48 h with 17β-estradiol (10^{-8} M) or EGF (10 ng/ml), in the presence or absence of an anti-c-Myc oligonucleotide ('antimyc', 10^{-6} M). DNA level and cAMP response to 10^{-8} M calcitonin were measured as described in Section 2). Results are mean $\pm$ S.D. ($n = 3$).

[a] $P < 0.01$ versus control.

prevent the effects of 17β-estradiol or EGF on DNA synthesis and on the cyclic AMP response of breast cancer cells to calcitonin (Table 1). These data suggest that the modulations exerted by the growth-regulating agents studied were not specific for the mitogen or the antimitogen used, but rather reflected their common ability to influence the position of breast cancer cells in the cell cycle.

3.4. Calcitonin receptor studies

We determined whether modifications at the level of calcitonin receptor might explain the variations in cyclic AMP response to the hormone after incubation of breast cancer cells with the growth-regulating agents. Scatchard analyses of radioiodinated calcitonin binding to T-47D cells did not reveal consistent variations in calcitonin receptor number or affinity. In one experiment, we found K_d values varying between 0.75 ± 0.07 nM (17β-estradiol) and 0.86 ± 0.11 nM ($1,25(OH)_2D_3$), while the apparent number of binding sites/cell varied between 28 100 (17β-estradiol) and 30 500 ($1,25(OH)_2D_3$). Another experiment gave K_d values between 0.66 ± 0.10 nM (tamoxifen) and 0.71 ± 0.11 (EGF) and a number of binding sites/cell between 26 600 ($1,25(OH)_2D_3$) and 29 900 (EGF), suggesting that neither changes in calcitonin receptor affinity or number could explain our findings.

3.5. Involvement of a pertussis toxin-sensitive G-protein

In many cells, a negative control of cyclic AMP production is mediated by a G-protein (G_i or G_o) that can be inactivated by pertussis toxin (Gilman, 1987). We tested whether the action of a pertussis toxin-sensitive protein could explain our results. As shown in Fig. 5, pertussis toxin (100 ng/ml) increased by about 1.7-fold ($P < 0.01$) the response of control cells to the hormone. Moreover, preincubation of breast cancer cells with the toxin almost completely suppressed the growth factors-induced differences in cyclic AMP response to calcitonin.

There was no effect of pertussis toxin on the breast cancer cells response to forskolin (data not shown).

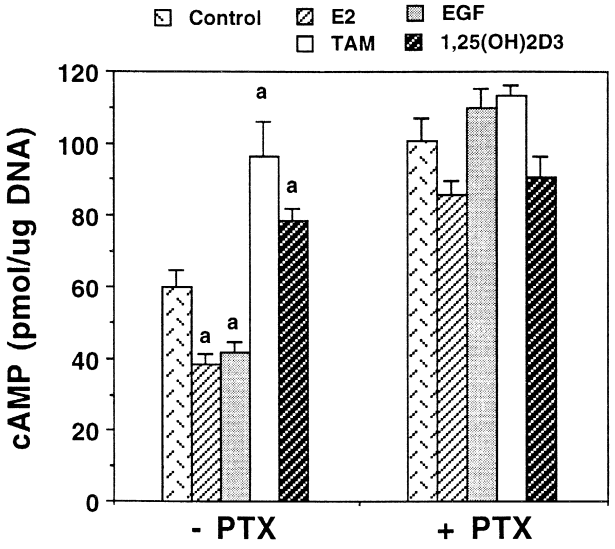

Fig. 5. Effects of 100 ng/ml pertussis toxin on the cyclic AMP response to calcitonin in T-47D breast cancer cells. Results are mean ± S.D. ($n = 4$); (a) $P < 0.01$.

Table 2
Effects of calcitonin and growth regulators on T-47D breast cancer cells proliferation

	DNA (μg/well)		Cells $\times 10^{-3}$/well
	Exp. 1	Exp. 2	
Control	4.3 ± 0.1	3.8 ± 0.1	235 ± 11
Control + CT	3.6 ± 0.1 (84%)[a]	3.3 ± 0.1 (88%)[a]	206 ± 10 (88%)[a]
E2	6.7 ± 0.2	6.1 ± 0.1	307 ± 8
E2 + CT	6.3 ± 0.2 (94%)	5.8 ± 0.2 (95%)	299 ± 8 (97%)
EGF	6.0 ± 0.2	5.4 ± 0.2	
EGF + CT	5.3 ± 0.1 (88%)[a]	4.7 ± 0.1 (88%)[a]	
TAM	3.0 ± 0.1	2.8 ± 0.1	195 ± 6
TAM + CT	2.2 ± 0.1 (74%)[b]	2.0 ± 0.1 (70%)[b]	134 ± 7 (69%)[b]
1,25(OH)2D3	3.6 ± 0.1	3.2 ± 0.1	
1,25(OH)2D3 + CT	3.1 ± 0.1 (86%)[a]	2.7 ± 0.1 (84%)[a]	
Antimyc			163 ± 6
Antimyc + CT			118 ± 6 (72%)[b]

T-47D cells were incubated for 4 days in the presence or absence of 10^{-8} M calcitonin and growth regulators (E_2, 10^{-8} M; EGF, 10 ng/ml; tamoxifen, 10^{-6} M; $1,25(OH)_2D_3$, 10^{-8} M; anti-Myc, 10^{-6} M). Results are expressed as μg DNA (two experiments: exp. 1 and exp. 2) or cell numbers (means of exp. 1 and 2) per well and are mean ± S.E. ($n = 4$).
[a] $P < 0.05$.
[b] $P < 0.01$
N.D.: not done.

3.6. Proliferation of T-47D breast cancer cells

Since cyclic AMP analogs have been reported to inhibit breast cancer cells growth (Cho-Chung, 1990), we tested if the antimitogenic action of calcitonin on T-47D cells could be modulated by agents that regulate the cyclic AMP response to calcitonin. Table 2 shows that calcitonin incubated alone on T-47D cells for 4 days led to a 12–16% decrease in breast cancer cells DNA and cell number but, in the presence of 17β-estradiol, calcitonin was devoid of significant growth inhibitory action. However, its antiproliferative action was enhanced when the hormone was given in association with tamoxifen or the anti-Myc oligonucleotide. In these latter cases, the DNA level and cell number were between 69 and 74% of specific controls in calcitonin-treated breast cancer cells. Co-addition of calcitonin and tamoxifen thus led to a 50% decrease in cell growth as compared to untreated cells ('control', first line of Table 2). In contrast, no significant modulation of calcitonin action was produced neither by $1,25(OH)_2D_3$ or EGF. No sign of significant cell death was seen in any condition, as assessed by the trypan blue exclusion test.

4. Discussion

We have shown here that the cyclic AMP response of breast cancer cells to calcitonin was decreased by mitogens and increased by growth inhibitors. Since these modulations were not influenced by the presence of the phosphodiesterase inhibitor IBMX, they proceeded essentially, if not exclusively, from changes in the cyclic nucleotide production.

Our data suggest that the calcitonin-induced cyclic AMP production was not modulated by the growth-regulating agents through changes in calcitonin receptor numbers or their affinity for the hormone. In contrast, those agents seemed able to modulate adenylate cyclase activity, another component of the calcitonin-signalling system. This is inferred from the fact that the cyclic AMP response to forskolin, a molecule known to directly activate the adenylate cyclase (Seamon and Daly, 1981), was modified, although to a lesser extent than seen with calcitonin, after incubation with the mitogens and antimitogens. The modulation of the adenylate cyclase could also modify its cyclic AMP production in response to the indirect activator calcitonin, partly explaining our observations. Eight different adenylate cyclases have been identified to date in vertebrates, each with its own sensitivity to intracellular modulators and its specific distribution in tissues and cell types (Iyengar, 1993). To our knowledge, no extensive investigation of the adenylate cyclase pattern of breast cancer cells has been performed and it remains to be determined whether mitogens and antimitogens might specifically regulate the amount of specific members of the adenylate cyclase-family. Such a modulation could be a secondary component of 17β-estradiol and tamoxifen action on cell growth, considering the antiproliferative effect of cyclic AMP and cyclic AMP inducers in breast cancer cells (Cho-Chung, 1990; Fortunati et al., 1996).

The cyclic AMP response of cells to isoproterenol is mediated by the β-adrenergic receptors (Emorine et al., 1989) coupled to the adenylate cyclase by at least one G-protein. That response was not significantly modulated by mitogens or antimitogens -with the notable exception of 17β-estradiol, which could act by increasing the number of β-adrenergic receptors (Marchetti et al., 1990). On the other hand, calcitonin and isoproterenol are not known to activate different species of adenylate cyclase. The relative specificity of the modulations that we observed with calcitonin was thus essentially associated with the molecule(s) coupling the calcitonin receptor to the adenylate cyclase, in all likelihood the G-protein(s).

Pertussis toxin almost abolished the growth modulators-induced changes in cyclic AMP response of T-47D cells to calcitonin. More precisely, the calcitonin-stimulated cyclic AMP concentrations in cells preincubated with 17β-estradiol, EGF or no agent were elevated by the toxin almost to the levels observed in cells preincubated with tamoxifen or $1,25(OH)_2D_3$. Although still controversial, an involvement of pertussis toxin-sensitive proteins (G_i/G_o proteins) in osteoclast response to calcitonin has been proposed by Zaidi et al. (1990). Chakraborty et al. (1991) showed that the cyclic AMP response to calcitonin of naturally calcitonin receptor-expressing, synchronized porcine kidney LLC-PK1 cells was very low in the S-phase of the cycle but increased to its G_2-phase value after preincubation of cells with pertussis toxin. These authors also observed no differences in calcitonin receptor number throughout the cell-cycle.

Our data thus appear to be in agreement with the model of a cell-cycle dependence for calcitonin receptor coupling to G-proteins. The two mitogens had similar effects on the cyclic AMP response of breast cancer cells to calcitonin; this was also the case for the two antimitogens. The effects were opposed between the two classes of agents. Moreover, the actions of 17β-estradiol and EGF were abolished by addition of the growth-inhibitory anti-*c-myc* oligonucleotide. It is known that estrogens and EGF increase the proportion of breast cancer cells in S-phase (Reddel and Sutherland, 1987; Wollman et al., 1994), while tamoxifen and $1,25(OH)_2D_3$ decrease it (Taylor et al., 1983; Saez et al., 1993; Maas et al., 1995). The hypothesis of a pertussis toxin action specifically restricted to breast cancer cells in S-phase might explain why the toxin affected essentially the cyclic AMP response of cells incubated with 17β-estradiol and EGF. A coupling has been proposed between the calcitonin receptor and a $G_{stimulatory}$ (G_s) protein, a protein frequently involved in cyclic AMP-generating cascades (Gilman, 1987). In breast cancer cells, our data obtained with the pertussis toxin suggest that the receptor could also be coupled to an as yet unidentified pertussis toxin-sensitive protein whose influence on adenylate cyclase could vary as a function of the position in the cell cycle, this latter being itself controlled by mitogens and antimitogens.

It is tempting to speculate that there is a direct link between the modulation of the cyclic AMP production in response to calcitonin and the changes in the growth inhibitory effects of calcitonin under the influence of 17β-estradiol, tamoxifen and the anti-c-*myc* oligonucleotide. The corresponding data obtained with $1,25(OH)_2D_3$ and EGF suggest, however, that the final effect on cell growth of a cocktail of mitogens and/or antimitogens may be the result of complex interactions between the different signals generated by these growth-controlling agents. Calcitonin could itself modulate the action of the other cell-growth regulators. For example, it has been shown that, in MCF-7 cells, cyclic AMP-producing agents can potentiate the estrogen receptor-mediated gene expression from an estrogen responsive element-containing promoter (Aronica et al., 1994); on the other hand, a prolonged exposure of MCF-7 cells to high cyclic AMP levels leads to a reduction in the number of estrogen receptors (Guilbaud et al., 1990). Concerning EGF, we have recently observed that its stimulation of the cell growth and induction of the cell

growth-associated proto-oncogene c-*jun* mRNA was prevented by calcitonin and forskolin in T-47D cells (Lacroix and Body, 1997). It, thus, appears that EGF and calcitonin may at least mutually attenuate some of their effects. Despite these restrictions, the present results suggest that some breast cancer cells-growth regulators (here 17β-estradiol and tamoxifen) might modify their activity by altering the signal transduction pathway of other apparently unrelated growth-modulatory molecules. It would be of interest to determine whether the inhibition of breast cancer cells growth by the cyclic AMP-inducing prostaglandins (Fentiman et al., 1984) is subject to the same alterations as those seen using calcitonin.

A number of breast cancer cells lines respond to calcitonin by an increase in cyclic AMP levels. Moreover, calcitonin receptor mRNAs are present in the mammary gland (Kuestner et al., 1994). Our data show that the cAMP response of breast cancer cells to calcitonin is inversely related to their growth rate. Further studies should investigate a possible action of calcitonin on breast cell differentiation and its synergistic activity with the growth inhibitory effect of anti-estrogens.

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