Modulation of HT-29 Human Colonic Cancer Cell Differentiation with Calmidazolium and 12-O-Tetradecanoylphorbol-13-acetate

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ABSTRACT

The effects of a protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), and of a calmodulin antagonist calmidazolium (CZ), on a human colonic cancer cell line HT-29 were analyzed. HT-29 cells are undifferentiated in standard culture conditions (HT-29 G+) and display an enterocytic differentiation when cultured in glucose-deprived medium (HT-29 G−). Early effects of TPA and CZ on the localization of cytoskeletal proteins (caldesmon, α-actinin and vinculin) and on cell proliferation were examined. Differentiation of the cells was assessed after 4 weeks on the basis of ultrastructural and functional characteristics of enterocytic polarity, presence of apical brush borders, expression of brush border membrane antigens (Caco 5/50 and sucrase-isomaltase), and segregation of calmodulin to the brush border cytoskeleton.

TPA treatment of HT-29 G+ or G− cells induced early morphological and cytoskeletal alterations: the cells rounded up and lost their stress fibers with the associated caldesmon, α-actinin, and vinculin. TPA did not modify the differentiation of G− cells, but induced in G+ cells the expression, although limited, of enterocytic differentiation characteristics. Addition of CZ to HT-29 G− cells enhanced their differentiation state but did not provoke any early morphological or cytoskeletal alterations. No effects of CZ on HT-29 G+ cells were obvious. The results suggest that protein kinase C, the TPA receptor, is involved in the triggering of HT-29 G+ cell differentiation whereas calmodulin-dependent functions would be implicated in HT-29 G− cell maturation.

INTRODUCTION

Enterocytes are highly polarized columnar epithelial cells, characterized by the presence at their luminal surface of thousands of microvilli forming the brush border (1, 2). Accumulating evidence suggests that in mature enterocytes, various cell functions are regulated by calcium ions (3). Indeed Ca2+ binds to two major proteins, the vitamin-D calcium binding protein named CaBP (4, 5) and calmodulin (5-7), both being concentrated in the brush border region. Upon binding calcium, calmodulin regulates the ionized calcium concentration in the microvilli (3), activates the actomyosin-based contractility system (8), and interacts with a number of actin-binding proteins (9). Calcium is also a direct cofactor of the phospholipid and Ca2+-dependent protein kinase C (10) which has been recently identified in intestinal epithelial cells (11).

Recently, much attention has been directed towards the assembly of the microvillous cytoskeleton during development (12-14) and in correlation with morphogenetic events (15). However, nothing is known concerning the mechanisms governing these processes. Among the mechanisms which could be responsible for microvillous cytoskeleton assembly and more generally for the biogenesis of enterocytes' polarity, a role for extracellular matrix components and epithelial-mesenchymal cell contacts has been suggested (1, 2, 15–17). Concerning the intracellular events, in many instances, calmodulin levels have been shown to regulate the differentiation processes (18, 19). Furthermore, activation of the protein kinase C may stimulate differentiation (20). Thus, in order to define the possible role of these two factors in enterocyte differentiation, we examined the effects of a calmodulin antagonist, CZ (21) and of a protein kinase C activator: the phorbol ester TPA (22). Indeed these compounds have been shown to mediate the terminal differentiation in various cell lines (for review see Reference 23), the most studied system being the human promyelocytic cell line HL-60 (24–27).

In order to examine terminal differentiation in human enterocytes an appropriate in vitro model was provided by the human HT-29 colon carcinoma cell line established by Fogh and Trempe (28). As shown by Zweibaum’s group (29–31), the state of differentiation of these cells is dependent on the culture conditions. Indeed HT-29 cells remain undifferentiated (HT-29 G+) when grown under standard conditions, in the presence of glucose. In contrast, when cultured in the total absence of sugar (HT-29 G−) they express a typical enterocytic differentiation starting after confluence (10–15 days) and being maximum after 30 days in culture (32). The morphological and enzymatic enterocytic features expressed by HT-29 G− cells remain however irregular and limited compared to small intestinal absorptive cells or to another colon adenocarcinoma cell line Caco-2 (32).

The present study examines the differentiation response of both HT-29 G+ and G− cells to the calmodulin antagonist CZ and to the protein kinase C activator, TPA. The early effects of TPA and CZ were studied on the basis of morphological and cytoskeletal alterations. The differentiation of the cells was assessed by ultrastructural criteria and by the expression and distribution of microvillous membrane and cytoskeletal antigens. The data reported herein emphasize that CZ increases the maturation of HT-29 G− cells whereas TPA triggers a "limited" differentiation of HT-29 G+ cells.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium was purchased from Eurobio (Paris, France). Fetal calf serum and trypsin were from Gibco Biocult (Glasgow, Scotland). Disposable plastic material used for cell culture experiments was from Falcon (Los Angeles, CA).

Benzamidine, antipain, pepstatin A, poly(ethylene oxide) 20 sorbitan monolaurate (Tween 20), inosine, phosphatidylycerine, and TPA were obtained from Sigma Chemical Co. (St. Louis, MO). Leupeptin, aprotanin, and calmidazolium were from Boehringer (Mannheim, W. Germany). Sodium dodecyl sulfate, acrylamide, N,N'-methylene-bis-acylamide were obtained from Bio-Rad Laboratories (Richmond, CA). NC membranes (0.45 µm) were purchased from Schleicher and Schuell (Dassel, W. Germany). Prestained molecular weight standards were from Bethesda Research Laboratories.

The abbreviations used are: CZ, calmidazolium; TPA, 12-O-tetradecanoylphorbol-13-acetate; NC, nitrocellulose; PBS, phosphate buffered saline; TCA, trichloroacetic acid.
from the Centre d’Energie Atomique (Saclay, France). Normal goat serum, fluorescein-conjugated goat anti-rabbit IgG, and fluorescein-conjugated rabbit antimouse IgG were obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands). Polyclonal calmodulin antibodies raised in rabbits were a generous gift of Dr. J. De Mey (Jansen Pharmacuetica, Beerse, Belgium); caldesmon antibodies were kindly provided by Dr. M. P. Walsh (Calgary, Canada). The mouse monoclonal Caco 5/50 antibodies (33) were kindly provided by Dr. A Quaroni (Cornell University, Ithaca, NY). Monoclonal antibodies against sucrase-isomaltase (HBB 2/614/88) (34) were kindly provided by Dr. H. P. Hauri (Biocenter, Basel, Switzerland). Monoclonals anti-α-actinin and antivinculin were from Bioweda and Biomekor (Kiryat Weizman, Rehovot, Israel), respectively.

Calmodulin was prepared as previously described (35) and iodinated using 125I-labeled Bolton and Hunter Reagent (2000 Ci/mmol, Amersham, les Ulis, France); the specific radioactivity of [125I]calmodulin ranged from 40 to 80 Ci/μg.

Cell Cultures and Cell Treatments. The human colon adenocarcinoma cell line HT-29 originally established by Fogh and Trempe (28) was obtained from Dr. A. Zweibaum (INSERM, U.178, Villejuif, France). The HT-29 G+ cells (passages 148 to 151) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and containing a standard concentration of glucose (25 mM). Stock cultures were subcultured by trypsinization (0.25% trypsin in Ca2+,Mg2+-free PBS containing 0.54 mM EDTA) and seeded at 1.8 x 10^4 cells/cm^2 in plastic dishes. The HT-29 G- cells (passages 6 to 12 following the switch of HT-29 G+ cells to sugar-free medium) were cultured in Dulbecco’s modified Eagle’s medium specially prepared without glucose by Eurobio and supplemented with 10% dialyzed fetal calf serum and 2.5 mM inosine. The cells were dissociated with 0.25% trypsin in Ca2+,Mg2+-free PBS containing 2.67 mM EDTA and seeded at 3.6 x 10^4 cells/cm^2.

At Day 2 (exponential phase of growth) CZ or TPA was added at concentrations of 0.5 μM and 10 nM, respectively. These concentrations were shown to be optimal by a dose-response (morphological behavior of the cells and [3H]thymidine incorporation) curve ranging from 0.05 to 1 μM for CZ and 1 to 100 nM for TPA (data not shown). Then the media supplemented with either CZ or TPA were changed daily. In control experiments, the cells were treated with the solvent (ethanol, 0.05%).

Morphological Analysis. For transmission electron microscopy, cell sheets scraped from the dishes were fixed in 0.2 M cacodylate-buffered 2% glutaraldehyde (pH 7.4) for 2 h at +4°C, postfixed in cacodylate-buffered 1% osmium tetroxide (pH 7.4) for 30 min at 4°C and embedded in araldite. Ultrathin sections were double stained with uranyl acetate and lead citrate before their observation with a Philips 300 electron microscope.

For scanning electron microscopy, cells were grown on plastic culture coverslips (Thermanox Lux Scientific Corporation, Newbury Park, PA). The cells were fixed as above, dehydrated, dried in a critical point drier, coated with gold, and examined with a Philips 501B scanning electron microscope.

[3H]Thymidine incorporation. [3H]Thymidine was added to the culture medium (1 μCi/ml) for a 24-h incubation at 37°C. Then, the radioactive medium was aspirated and the culture rinsed twice with PBS and trypsinized. After centrifugation, aliquots of the cell suspension were spotted onto discs of Whatman paper which were successively treated with TCA 20% (twice for 5 min), TCA 10% (twice for 5 min), ethanol (5 min), ethanol/ether (3 min), and ether (twice for 2 min). The discs were then dried and the adsorbed radioactivity measured in an Inter-

Fig. 1. Phase contrast micrographs of HT-29 G+ (a, c, e) and G- (b, d, f) cells cultured for 3 days. a, b, control cells; c, d, CZ (0.5 μM)-treated cells; e, f, TPA (10 nM)-treated cells. CZ and TPA were added 2 days after plating and cell morphology was examined 24 h later. Bar, 50 μm.
Fig. 2. Effect of TPA on caldesmon, α-actinin, and vinculin distribution in HT-29 G+ cells. Cells were grown on glass coverslips and stained as described in "Materials and Methods" after a 24-h treatment with 0.05% ethanol (a, d, g), 10 nM TPA (b, e, h) or 0.5 μM CZ (c, f, i). Bar. 50 μm. a, h, c, caldesmon; d, e, f, α-actinin; g, k, i, vinculin.

technique Scintillation Counter. The rate of [3H]thymidine incorporation was expressed as dpm/24 h/mg of protein.

Immunofluorescence Microscopy on Cryosections. This technique was used to demonstrate the expression and localization of specific brush border antigens (Caco 5/50, sucrase-isomaltase, calmodulin). After washing the cells twice in Hanks’ buffer, a “sheet” of cells was collected, fixed for 1 h at 4°C with 2% paraformaldehyde in 0.1 M piperazine-1,4-bis-2-ethanesulfonate at pH 7.0 containing 5% sucrose and embedded in Cryoform. Cryosections (4–5 μm thick) were cut with a cryostat and incubated with the specific mouse CaCo 5/50 (33) or sucrase (34) monoclonals or with the polyclonal rabbit calmodulin antibodies (36). The second antibodies were fluorescein-conjugated rabbit anti-mouse and goat anti-rabbit sera, respectively. Sections were mounted in phosphate-buffered glycerol supplemented with paraphenylenediamine (37) and observed using a Leitz epifluorescence microscope. Controls included incubations without monoclonals or after neutralization of calmodulin antibodies with native calmodulin (35, 36).

Immunofluorescence on Cultured Cell Monolayers. This immunofluorescence technique was used to localize caldesmon, α-actinin, and vinculin. Cells routinely cultured on glass coverslips were rinsed three times in Tris buffered saline, fixed for 10 min in methanol at −20°C, and permeabilized in cold acetone (10 min). Then the cultures were processed for indirect immunofluorescence as described for cryosections. The second antibodies were goat anti-rabbit IgG conjugated to fluorescein for caldesmon, and fluorescein-conjugated rabbit anti-mouse IgG for α-actinin and vinculin.

Preparation of Cultured Cell Lysates. Cultured cells were washed twice in ice-cold Ca2+,Mg2+-free Hanks’ balanced salt solution (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.3 mM Na2H PO4, 0.4 mM KH2PO4, 140 mM NaCl, 5.4 mM KCl, pH 7.3) containing 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid and a cocktail of protease inhibitors [pepstatin (1 μg/ml), antipain (1 μg/ml), benzamidine (15 μg/ml), leupeptin (10 μg/ml), and aprotinin (10 μg/ml)]. Cells were then scraped in Hanks’ buffer, sonicated, and stored at −80°C before analysis.

Determination of Calmodulin Content. The cell lysates were treated at 80°C for 5 min, rapidly cooled on ice, and centrifuged at 8000 × g for 5 min. Then the supernatants were assayed for calmodulin content using a commercially available [125I]calmodulin radioimmunoassay kit from New England Nuclear (Frankfurt, Germany). Results were expressed as the mean of six replicates in ng/mg protein. Proteins were estimated according to Lowry et al. (38).

Detection of Calmodulin-binding Proteins. Proteins from cell lysates were resolved by electrophoresis in 5–15% linear gradient polyacrylamide gels (0.75 mm thickness) in the presence of 0.1% sodium dodecyl
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RESULTS

Cell Morphology and Cytoskeleton Organization after CZ and TPA Treatment. HT29 G+ and G− cells grow normally as tight colonies of polygonal cells (Fig. 1, a and b). Their treatment in exponential phase of growth (Day 2 after seeding) with CZ (0.05–0.5 μM) for several days did not result in any change in morphology when examined under the phase contrast microscope (Fig. 1, c and d). However, they showed a clear response to TPA (1–100 nM) as soon as 24 h (Fig. 1, e and f): the colonies extended and the cells rounded up showing large intercellular cysts bordered with microvilli are found within the upper surface of approximately 50% of the culture (Fig. 3). Treatment of G+ cells with TPA (10 nM) led to opposite effects of CZ and TPA on HT-29 Cell Differentiation. The ultrastructural features of both HT-29 G+ and G− cells viewed at the transmission or scanning electron microscope have already been described (32). Briefly, HT-29 G+ cells display 3–4 weeks of culture, multilayers of polymorphic cells with sparse and irregular cytoplasmic processes (Figs. 4a and 5a). In contrast, grown in the absence of glucose, HT-29 G− cells form a monolayer of polarized polygonal cells linked by apical junctions. They exhibit at their apex numerous true microvilli projecting towards the medium (Figs. 4b and 5b). In addition, intercellular cysts bordered with microvilli are found within the cell layer of either G+ or G− cells (Fig. 4, a and b).

When CZ (0.5 μM) was present for 4–5 weeks in the culture medium of the HT-29 G+ cells, no change in the ultrastructural characteristics of these cells could be observed (Figs. 4c and 5c). However, CZ caused important modifications in G− cells. Fig. 4d shows that CZ-treated HT-29 G− cells depicted more dense and more regular microvilli uniformly distributed at their apical surface and exhibiting distinct cytoskeletal rootlets. Treatment of HT-29 cells with TPA (10 nm) led to opposite results. Indeed, TPA did not modify the ultrastructural pattern of the differentiating G− cells (Fig. 4f). However after 4 weeks, TPA-treated HT-29 G+ cells, although still growing in multilayers exhibited numerous and dense organized microvilli at the upper surface of approximately 50% of the culture (Fig. 5d), the remaining cells facing the medium being not significantly different from the control ones (Fig. 4e).

Thus, from these results it appears that the effects of CZ and TPA on the ultrastructural characteristics of HT-29 cells differ depending on the differentiation potential of these cells. It must

Fig. 3. Effect of CZ and TPA on [³H]thymidine incorporation of HT-29 G+ and G− cells. CZ (0.5 μM) and TPA (10 and 100 nm) were added as Day 2 after plating. [³H]Thymidine was added for a 24-h incubation at the time of treatment and 2 days later as described in “Materials and Methods.” Results represent the mean ± SD of two experiments performed in duplicate.

sulfate (39) and electrophoretically transferred to NC membranes as already described (40). The calmodulin-binding proteins bound to NC membranes were visualized by [¹²⁵I]calmodulin overlay as already reported (13).

Determination of Sucrase Activity. Sucrase activity was measured according to Messer and Dahlqvist (41) in a fraction enriched in brush border membranes (42). The results are expressed as milliunits per mg of proteins. One unit is defined as the activity that hydrolyzes 1 μmol of sucrose per min under the experimental conditions.

Effects of CZ and TPA on HT-29 Cell Proliferation. When undifferentiated HT-29 G+ cells treated at Day 2 after seeding with 0.5 μM CZ, their growth rate was 50% reduced as assessed by the decrease of [³H]thymidine incorporation during the 24 h following (Fig. 3). Treatment of G+ cells with 10–100 nM TPA caused a similar inhibition of cell growth. In contrast, when added to HT-29 G− cells, both TPA and CZ acted as stimulators of [³H]thymidine incorporation (50–150%). These responses, detectable as soon as 24 h of treatment, were still visible after 72 h. Thus, in the HT-29 cell line, TPA and CZ acted either as growth inhibitors or as mitogens, depending on the differentiation potentiality of the cells linked to their culture conditions.

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be stressed that these CZ- and TPA-induced changes in the differentiation state of G− and G+ cells, respectively, occurred only when the compounds were present in the medium during the whole culture period (4–5 weeks). Indeed a single 24-h exposure of growing cells to CZ or TPA was ineffective on these late effects. Moreover the effects of CZ and TPA on the differentiation of HT-29 cells were maintained during several passages when the cells were continuously cultured in their presence but were reversible if the cells were subcultured in control medium.

Effects of CZ and TPA on Sucrase-Isomaltase Immunofluorescence. As already demonstrated by Zweibaum’s group (30), the differentiation of HT-29 G− cells was proved by the expression of an intestinal microvillar hydrolase, sucrase-isomaltase: the enzyme is detectable at the apical surface of 50–60% of the cells (31) as shown by immunofluorescence with monoclonal anti-sucrase-isomaltase antibodies (Fig. 6b). No positive staining could be detected in the pluristratified undifferentiated G+ cells (Fig. 6a).

As expected from the above mentioned results, CZ did not modify the negative immunofluorescence pattern of G+ cells (Fig. 6c) but increased the number of G− cells labeled at their apex (Fig. 6d). On the other hand, TPA did not modify the sucrase-isomaltase immunofluorescence staining of G− cells (Fig. 6f). However, Fig. 6e shows that surprisingly, TPA did not induce the appearance of any labeling in G+ cells.

These immunofluorescence results were confirmed by determination of sucrase activity in brush border membrane fractions: CZ increased sucrase activity in G− cells after 4 weeks of culture (13.3 mU/mg protein) as compared to controls (7.5
mU/mg protein), whereas TPA was ineffective; neither CZ nor TPA was able to induce sucrase activity in G+ cells.

Immunofluorescence of the Caco 5/50 Antigen. As another approach, the differentiation of HT-29 cells has been studied by immunofluorescence of the Caco 5/50 antigen, using specific monoclonal antibodies. The Caco 5/50 antibodies recognize exclusively an antigen located at the surface membrane in human jejunum epithelial cells as well as in the spontaneously differentiated human colonic cancer cell line Caco-2 (33). As shown in Fig. 7d, these antibodies stained, although discontinuously, the apical surface of HT-29 G— cells after 4 weeks of culture. These cells also exhibited some strongly positive intercellular cysts already visible around 10 days of culture (Fig. 7b). In contrast, the pluristratified undifferentiated HT-29 G+ cells did not depict any Caco 5/50 staining up to 4–5 weeks of culture (Fig. 7, a and c).

The presence of CZ during 4 weeks did not modify the negative immunofluorescence pattern of HT-29 G+ cells (Fig. 7e). However CZ increased strongly the number of G— cells labeled at their apex, the interruptions of the apical staining corresponding to morphologically less differentiated cells (Fig. 7f). TPA treatment, in contrast to CZ, did not modify the Caco 5/50 apical staining of G— cells (Fig. 7h) but induced the staining of some intercellular cysts in HT-29 G+ cells (Fig. 7g).

Effects of CZ and TPA on Calmodulin Levels and Localization. To determine whether calmodulin was of regulatory significance during HT-29 cells' differentiation, calmodulin levels were measured by radioimmunoassay in growing and post-confluent HT-29 G+ and G— cells submitted or not to CZ or TPA treatment. As shown in Fig. 8, 2 days after seeding, both HT-29 G+ and G— cells exhibited low calmodulin concentrations. Then calmodulin increased progressively, reaching maximal values at confluency for HT-29 G+ cells and later (around 3 weeks) for G— cells. 24 h after their addition, both CZ and TPA decreased calmodulin concentrations in G+ cells (respectively, 29.9 ± 7.7 and 33.5 ± 6.1 ng/mg protein) as compared to controls (71.7 ± 7.5 ng/mg protein). These effects were no more obvious after 4 weeks. However, neither CZ nor TPA did modify calmodulin concentrations of HT-29 G— cells up to 4 weeks of culture.

Since it had been previously demonstrated that in rats and humans (14, 36, 43) the differentiation of intestinal epithelial cells was accompanied by the segregation of calmodulin to their apical side, we also studied by immunofluorescence on cryosections the effects of CZ and TPA on calmodulin distribution.
Fig. 6. Immunofluorescent staining of HT-29 G+ (a, c, e) and G− (b, d, f) cell sheets with monoclonal antibodies specific for sucrase-isomaltase after 4 weeks of culture in control medium (a, b) or in the presence of 0.5 mM CZ (c, d) or 10 nM TPA (e, f). Note that CZ increased the apical labelling of HT-29 G− cells (d). It should be noted that some HT-29 G+ cells (a, c, e) show some aspecific autofluorescence which is easily distinguished from the greenish specific immunoreactions by its yellowish color. Bar, 50 μm; arrows point to the apical staining.

Fig. 7. Immunofluorescent staining of HT-29 G+ (a, c, g) and G− (b, d, h) cells with the monoclonal antibodies Caco 5/50. a, b, control cells after 10 days of culture; c, d, control cells after 30 days of culture; e, f, cells cultured for 4 weeks in the presence of 0.5 mM CZ; g, h, cells cultured for 4 weeks in the presence of 10 nM TPA. Note in TPA-treated HT-29 G+ cells (g) the appearance of a positive labelling in intercellular cysts, and in CZ-treated G− cells (f) the presence of a more regular apical staining as compared to controls (d). It must be stressed that the apparent staining in e is aspecific. Bar, 50 μm.

Fig. 9a demonstrates that in undifferentiated HT-29 G+ cells up to 4 weeks of culture, calmodulin staining was weak and lined the periphery of individual cells. However in HT-29 G− cells, in addition to the peripheral staining of the cells, calmodulin fluorescence had segregated to the apical side of the differentiated cells (Fig. 9b). Neither CZ nor TPA treatments led to modifications in the pericellular calmodulin immunofluorescence pattern of HT-29 G+ cells (Fig. 9, c and e). However the apical staining of HT-29 G− cells was increased by CZ (Fig. 9d) whereas it was unmodified by TPA (Fig. 9f).

Calmodulin-binding proteins were also investigated after CZ and TPA treatment. Both HT-29 G+ and G− cells contain three major calmodulin binding proteins with apparent molecular weights of 240,000, 145,000 and 135,000 which have been previously characterized as fodrin, caldesmon, and an immunoreactive form of the M, 110,000 protein, respectively (13, 14, 44). Neither CZ nor TPA appeared to alter this pattern of calmodulin-binding proteins in G+ as in G− cells (data not shown).

DISCUSSION

The present study examined possible roles of calmodulin and protein kinase C in the differentiation of HT-29 colonic adenocarcinoma cells by using the calmodulin antagonist calmidazolium and the protein kinase C activator TPA. The HT-29 cell line provides a good system for investigating the differentiation processes since these cells are able to undergo differentiation and to express structural and functional features of differentiated enterocytes when grown in glucose-free medium (30). The message conveyed by the present report is that CZ and TPA act differentially on HT-29 cells depending on the differentiation potential of these cells that is associated with particular culture conditions: CZ favors the enterocytic differentiation pathway of HT-29 G− cells cultured in the absence of sugar whereas TPA triggers a limited differentiation of HT-29 G+ cells which in the standard culture conditions (presence of 25 mM glucose) remain undifferentiated.

The action of CZ on HT-29 G− cell differentiation was visualized by increases (a) in the density of microvilli at the cell surface with well-organized cytoskeletal rootlets, (b) in the apical Caco 5/50 antigen and sucrase-isomaltase expression, and (c) in calmodulin apical segregation. It must however be emphasized that the differentiation degree of CZ-treated HT-29 G− cells remains far lower than that described in the spontaneously differentiating Caco-2 cells (32). The effects of CZ on HT-29 G− cells corroborate other data which demonstrated that calmodulin antagonists were able to augment differentiation processes of HL-60 cells (27).

The action of TPA on exponentially growing HT-29 G+ cells resulted in early morphological and cytoskeletal alterations...
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Fig. 8. Growth-related calmodulin concentrations (ng/mg protein) in G+ (O- -O) and G- (● - ●) HT-29 cells. At the appropriate time points, calmodulin content was determined by radioimmunoassay as described in "Materials and Methods." The values presented herein are the mean ± SD of two dishes assayed in triplicate.

Fig. 9. Immunofluorescent staining of G+ (a, c, e) and G- (b, d, f) HT-29 cells with calmodulin antibodies after 4 weeks of culture in the presence of 0.05% ethanol (a, b), 0.5 μM CZ (c, d) or 10 nM TPA (e, f). Bar, 50 μm.

Disappearance of microfilament stress fibers and re-arrangement of α-actinin and vinculin as already described in other cell culture systems (45, 46) followed after 4 weeks by ultrastructural changes of the cells. The most obvious finding was the appearance of organized polygonal cells with dense and regular microvilli at their apical surface. These phenotypic characteristics were accompanied by the appearance of the Caco 5/50 antigen in the intercellular cysts, the apex of the cells facing the medium being always devoid of staining. It must be stressed that other known differentiation criteria of HT-29 cells were not observed: the cell layer remained pluristratified and did not display any sucrase-isomaltase either at the apex or in the intercellular cysts. Furthermore, calmodulin did not segregate to the apex of the cells. Thus, these observations suggest that TPA triggers only a limited expression of differentiation characteristics in G+ cells. Intercellular cysts within the stratified cell layers correspond to the organization of a small number of cells surrounding a lumen which displays apical membrane features. It has been proposed that these formations are involved in the establishment of cell polarity (47, 48). The observation that Caco 5/50 antigen is found in TPA-treated HT-29 G+ cells exclusively within the cysts, while other brush border antigens such as sucrase-isomaltase are not expressed, emphasizes the interest of this Caco 5/50 antigen as an early marker of intestinal cell differentiation. It must be stressed that induction of HT-29 G+ cell differentiation has already been obtained with other components such as sodium butyrate (49). However, while this latter agent triggered the emergence of permanently differentiated cell clones, TPA effects remained reversible. The effect of TPA on the differentiation of HT-29 intestinal cancer cells is interesting since up to now a TPA-induced differentiation was described only in leukemia cells (23–26).

In conclusion, both CZ and TPA act as differentiating agents. However, their mechanism of action may be different. Although CZ, like all calmodulin antagonists, may have noncalmodulin-related effects, our results are consistent with the hypothesis that inhibition of a calmodulin-mediated function (without alterations in calmodulin concentrations nor in the nature of calmodulin-binding proteins) may augment the differentiation potential of HT-29 G- cells.

The early morphological and cytoskeletal alterations observed in HT-29 G+ cells after TPA treatment do not seem to be related to the differentiating action of TPA since this phorbol ester provoked the same rapid changes in G- cells, whose differentiation was unaffected. Furthermore, the observation that CZ did not induce any cytoskeletal response before increasing the maturation of HT-29 G- cells strengthens the hypothesis according to which these early alterations are not involved in the differentiation processes. The "differentiating" action of TPA on HT-29 G+ cells was preceded by a reduction of their growth rate. However, the fact that CZ acted similarly without modifying the ultrastructural characteristics of these cells suggests that their differentiation is not linked to a lowering of the growth rate. The same conclusion could be afforded for the rapid drop in calmodulin levels which rather appears to be related to the antiproliferative effect of TPA as well as of CZ.

Recently, phosphorylations have been considered as possible regulatory events of differentiation (50–52). According to preliminary results, HT-29 G+ and G- cells exhibited proteins whose phosphorylation intensity was increased early after TPA and CZ treatment, respectively. Although the nature of these phosphorylatable substrates remains to be determined, these observations suggest that phosphorylation processes may be involved in the mechanisms by which TPA and CZ modulate HT-29 cell differentiation. The prevailing current hypothesis is that these agents exert their biological effects by inducing an altered program of gene expression, a process that involves protein kinase C, as recently demonstrated for TPA (53). In

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Unpublished data.
conclusion, this study of HT-29 G+ and G— cell differentiation offers the opportunity to investigate, at the molecular level, which pathway is responsible for transducing the signals generated by TPA and/or CZ from the plasma membrane to the transcriptional machinery. Thus, the human colon cancer cell line HT-29 provides a useful system to investigate whether such differentiating agents could be envisaged in colon cancer chemotherapy strategies.

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