0013-7227/02/$15.00/0
*Printed in U.S.A.*

Endocrinology 143(10):4018–4030

doi: 10.1210/en.2002-220200

# Antitumorigenic and Antiinsulinogenic Effects of Calcitriol on Insulinoma Cells and Solid β-Cell Tumors

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**Malignant insulinoma is a rare form of cancer with a poor prognosis because of metastatic dissemination and untreatable hypoglycemia. Effective chemotherapy of patients who are not cured by surgery is needed. Calcitriol has known anticancer properties on different neoplastic cell lines, but no data are available regarding its activity on tumorigenic pancreatic β-cells. We analyzed the *in vitro* effects of calcitriol on the murine insulinoma cell line $\beta TC_3$ and primary cultures of human isolated islets and benign insulinoma. The effect of *in vivo* calcitriol administration on insulinoma of recombinant insulin/Simian virus 40 oncogene-expressing transgenic mice was also investigated. In $\beta TC_3$, calcitriol induced growth inhibition; apoptosis; down-regulation of insulin gene expression; and nongenomic activation of the MAPK pathway. MAPK kinase inhibitor (UO126) and staurosporine reduced calcitriol-mediated $\beta TC_3$ death, and down-regulation of insulin gene transcription was prevented by staurosporine but not UO126. Calcitriol significantly decreased insulin release and mRNA levels of human islets and insulinoma cells. Finally, recombinant insulin/Simian virus 40 oncogene-expressing transgenic mice treated with calcitriol showed reduced insulinoma volumes because of increased apoptosis of adenomatous cells. Together, these findings provide the rationale for testing the efficacy of calcitriol in the treatment of patients with solid β-cell tumors. (*Endocrinology* 143: 4018–4030, 2002)**

CALCITRIOL, THE HORMONALLY active form of vitamin $D_3$ [1,25-$(OH)_2D_3$], regulates the transcription of a variety of genes, with important effects on calcium homeostasis, regulation of the immune response, and cell differentiation (1, 2). Over the past decade, it has become evident that calcitriol and its analogs also exhibit antiproliferative activities in a variety of tumorigenic cell lines (3–9), raising the possibility that calcitriol, besides its classical uses in secondary hyperparathyroidism and psoriasis, might be employed in the treatment of malignancies (10). Malignant insulinoma is a rare cancer with a poor prognosis and a reported 5-yr survival rate of 35% (11). In contrast to benign β-cell adenomas, which are usually cured by surgery, malignant insulinomas are often diagnosed when metastatic dissemination has already occurred so that surgical resection is rarely successful (12). Several cytotoxic agents have been employed for chemotherapy of malignant insulinomas but with disappointing results (13). In addition to metastatic dissemination, untreatable hypoglycemia, consequent to unregulated insulin release by neoplastic β-cells, is a life-threatening condition in these patients. In contrast to the hypoglycemia associated with benign insulinomas, which is well controlled by inhibitors of insulin release such as diazoxide and certain calcium channel antagonists, malignant insulinomas are usually unresponsive to these drugs. Moreover, somatostatin and its long-acting analogs are effective in controlling hormone-mediated symptoms and growth of functionally active neuroendocrine tumors of the gastrointestinal tract but are usually ineffective in the treatment of malignant insulinomas (14).

The presence of nuclear vitamin D receptors (VDRs) in the pancreatic β-cell has been demonstrated (15, 16), but the precise role of calcitriol in islet cell physiology is still unclear. Vitamin D-deficient rats show an impaired insulin response to a glucose challenge (17, 18), and adverse effects of vitamin D deficiency on insulin secretion have also been reported in man (19, 20). These effects can be corrected by calcitriol administration (19, 20), and the beneficial effects of vitamin D may actually depend on its capability to raise plasma calcium concentrations (21). The beneficial effects of calcitriol on insulin secretion have been documented by a large series of *in vitro* studies, mostly on islets isolated from vitamin D-depleted rats. Conversely, an inhibitory effect of long-term calcitriol exposure on β-cell growth and insulin secretion has been reported in islets from vitamin D-repleted rats and the rat insulinoma cell line RIN-38 (22). However, surprisingly little is known about the effects that calcitriol exerts on islets isolated from normal animals.

Studies on the relationship between vitamin D and pan-

Abbreviations: BrdU, 5′-Bromo-2′deoxyuridine; DAPI, 4′,6′-diamino-2-phenylindole; FCS, fetal calf serum; LDH, lactate dehydrogenase; MEK, MAPK kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDX-1, pancreatic duodenal homeobox-1; PKC, protein kinase C; RIP1Tag2, recombinant insulin/Simian virus 40 oncogene-expressing transgenic mice; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling; VDR, vitamin D receptor.

creatic β-cells have become even more appealing after the demonstration that calcitriol and its analogs can delay and reduce the onset of diabetes in the nonobese diabetic mouse (23, 24) and that a polymorphism within the vitamin D receptor gene may play a role in the pathogenesis of either type 1 (insulin-dependent) and type 2 diabetes mellitus (25–27).

Despite its known anticancer properties, no studies have been previously conducted to test the antineoplastic activity of calcitriol on tumorigenic β-cells. In the present study, we have examined the effects of calcitriol *in vitro* on the mouse β-cell line $\beta TC_3$ (28) and *in vivo* in the recombinant insulin/Simian virus 40 oncogene-expressing transgenic mice (RIP1Tag2) from which $\beta TC_3$ cells were derived (29). RIP1Tag2 mice inheritably develop insulinomas with a precise multistep model of tumorigenesis. Islets are essentially normal in newborn mice and become hyperplastic in young adult mice (4–6 wk). By the age of 9 wk, 50% of the islets are composed of proliferating β-cells (29, 30), but only a few islets progress to histologically distinct adenomas, which are present by 12 wk of age in all mice of this lineage. Before becoming adenomas, hyperplastic islets start to produce angiogenic factors that are necessary for the transition from hyperplasia to neoplasia (31). If left untreated, RIP1Tag2 mice die of hypoglycemia 12–16 wk after birth; however, if glucose is added to their drinking water, they survive long enough to show the progression of a few benign insulinomas into invasive tumors. The transition from well-differentiated adenomas to carcinomas coincides with the lack of the adhesion molecule E-cadherin in adenomatous cells (32). Moreover, neural cell adhesion molecule-deficient RIP1Tag2 mice develop metastases that are never observed in normal RIP1Tag2 mice (33).

Our results show that calcitriol has a profound antineoplastic effect on insulinoma cells, both *in vitro* and *in vivo*. In addition, calcitriol significantly decreases gene expression, total content, and release of insulin both in mouse and human insulinoma cells as well as in isolated human islets. The implications of these findings for future trials aimed at determining the efficacy of calcitriol or its analogs in the treatment of patients with solid β-cell tumors are discussed.

## Materials and Methods

### *Cell lines and cultures*

$\beta TC_3$ cells were provided by Shimon Efrat (Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel); $\alpha TC_1$ cells from Douglas Hanahan (Department of Biochemistry and Biophysics, University of California, San Francisco, CA), and pituitary $GH_3$ from the American Type Culture Collection (Manassas, VA). $\beta TC_3$ cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 IU/ml streptomycin/penicillin. The $\alpha TC_1$ and $GH_3$ cells were cultured in DMEM (Life Technologies, Inc.) containing 25 mM glucose and supplemented with 10% FCS, 2 mM glutamine, and 100 IU/ml streptomycin/penicillin. Cultures were performed under standard humidified conditions of 5% $CO_2$ and 95% air at 37 C.

### *Preparation and culture of human insulinoma cells*

Insulinoma tissue was obtained from a 34-yr-old male who was admitted to the hospital for hypoglycemia and hyperinsulinism. At the operating field, the adenoma appeared as a nodule of 15 mm in diameter, easily excisable from the surrounding pancreatic parenchyma. After removal, the tumor was divided into two parts; a part was sent to the Pathology Department for routine histology that, according to the criterion for the classification of the endocrine tumors (34), confirmed the clinical diagnosis of benign insulinoma. The other part was taken to the laboratory in which it was minced into small fragments and then dispersed into a suspension of cell clusters by vigorous pipetting. After washing, insulinoma cells were plated in DMEM/F12 medium containing 2 mM L-glutamine, 33.2 mM glucose, 9.6 μg/ml putrescine, 6.3 ng/ml progesterone, 5.2 ng/ml sodium selenite, 0.025 mg/ml insulin, 0.1 mg/ml transferrin, and 2 μg/ml heparin (sodium salt, grade II, Sigma, St. Louis, MO) onto 100 × 20-mm plastic culture dishes and allowed to recover for 3–5 d before being used for the experiments. Medium changes were performed every 48 h.

### *Isolation and culture of human islets*

Human pancreatic islets were isolated from the pancreases of cadaveric multiorgan donors by using the procedure already described in detail (35). The islets used in this study were isolated from eight different organs. After the isolation, the islets were purified from the contaminant exocrine tissue by centrifugation on Ficoll gradients, obtaining a final purity that ranged from 60% to 80%, as assayed by dithizone staining (35). Islets were then cultured in RPMI 1640 tissue culture medium. After a 48-h stabilization culture, old medium was replaced with fresh medium containing vehicle or increasing calcitriol (kindly provided by Hoffmann-La Roche Inc., Nutley, NJ) concentrations (10, 100, and 1000 nM), and islets were cultured for an additional 48 h until used for insulin secretory studies or RNA extraction.

### *Cell growth in vitro*

Cell proliferation was determined in $\beta TC_3$, $GH_3$, and $\alpha TC_1$ cell lines. The cells were seeded at a density of $9 \times 10^4$ cells per well onto 24-multiwell culture plates. After allowing the cells to attach and growth for 48 h, cells were treated with vehicle (ethanol, final concentration 0.04%) or increasing calcitriol concentrations (10, 100, and 1000 nM) dissolved in ethanol. After 48 and 72 h of vehicle or calcitriol exposure, cells were harvested and counted in duplicate with a Coulter Counter ZM (Beckman Coulter, Inc., Fullerton, CA).

### *Cell cycle experiments*

Analysis of cell cycle was performed on $\beta TC_3$ and $GH_3$ cells that were plated at the density of $1.8 \times 10^6$ cells into 60-$cm^2$ tissue culture plates and allowed to attach and grow in standard medium for 48 h. On the third day, cells were refed with fresh medium with vehicle or calcitriol (10, 100, and 1000 nM) and cultured for an additional 48 h. Before harvesting, cells were pulse labeled with 10 μM 5′-bromo-2′deoxyuridine (BrdU, a thymidine analog that is incorporated into newly synthesized DNA by the cells entering and progressing through the S phase of the cell cycle) for 15 h. Cell cycle distribution was determined by the BrdU flow kit (PharMingen, San Diego, CA). The incorporated BrdU was stained with specific anti-BrdU fluorescent antibodies, and positive cells were detected and counted by flow cytometry. The phase of cell cycle and DNA synthetic activities of the cells were determined by analyzing the correlated expression of total DNA (7-AAD) and incorporated BrdU levels. Different region gates were applied and the percentage of the cells in the different cell cycle compartments was measured according to the kit's manufacturer instructions.

### *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay*

The number of viable cells was assayed using the MTT (Sigma) method. Vital dye staining was performed on $\beta TC_3$, $\alpha TC_1$, and $GH_3$ cells that were seeded at a density of $3 \times 10^4$ cells per well onto 96-well culture plates and allowed to attach and grow for 48 h in standard medium. On the third day, cells were washed and refed with fresh medium containing vehicle or increasing concentrations of calcitriol (10, 100, and 1000 nM). Number of viable cells was measured after 48 and 72 h as previously described (36).

### *Detection of apoptosis and quantification of fragmented DNA*

Detection of apoptosis and quantification of fragmented DNA was performed in $\beta TC_3$ cells and primary cultures of the benign insulinoma. The $\beta TC_3$ cells were seeded at a density of $2 \times 10^6$ onto 25-$cm^2$ tissue culture flasks and allowed to attach and grow for 48 h. On the third day, old medium was replaced with fresh medium containing vehicle or increasing calcitriol concentrations. After 48 h cells were harvested, lysed in 100 μl lysis buffer (10 mM Tris-Hcl; 10 mM EDTA; 0.5% Triton X-100, pH 8.0), and centrifuged at 10,500 rpm for 5 min. Supernatants containing fragmented (soluble) DNA were collected and pellets containing insoluble DNA were further extracted; both DNA fractions were then treated in sequence with Rnase and proteinase-K for 1 h and precipitated with isopropanol. Pellets were then dissolved in Tris/EDTA and DNA concentrations were measured by spectrophotometer. Fragmented DNA was calculated as 100% × soluble DNA/total DNA. DNA ladders were visualized by agarose/ethidium bromide gel electrophoresis. Apoptosis was also detected by immunofluorescence staining of βTC3 cells and human islet treated with calcitriol 10, 100, and 1,000 nM. Cells monolayers were fixed in freshly prepared 4% paraformaldehyde (in 0.05 M PBS, pH 7.4, Life Technologies, Inc.) for 10 min at room temperature and then immunostained for insulin (mouse antihuman insulin, diluted 1:100, Signet Laboratories, Dedham, MA) and 4′,6′-diamino-2-phenylindole (DAPI, 1 mg/ml in 1× PBS, diluted 1:50, Sigma) for determination of nuclear morphology.

Primary cultures of human insulinoma cells were treated with 0.05% trypsin in 0.05 mM EDTA at 37 C for about 5 min. Detached cells were washed twice, seeded again at a density of $5 \times 10^4$ cells onto a chamber slide with a cover (Nalge Nunc International, Rochester, NY), and allowed to form cell monolayers for 48–72 h in standard medium. On the day of the experiments, the old medium was discarded and replaced with fresh medium containing vehicle or calcitriol (100 nM and 1000 nM) and culture continued for additional 48 h. Detection of apoptosis was performed by fluorescence microscopy as described for $\beta TC_3$ and human islets. Monolayers were immunostained for insulin for pancreatic duodenal homeobox-1 (PDX-1) (rabbit antihuman, diluted 1:2000) and nuclear morphology by DAPI. The number of insulin-positive cells showing condensed or fragmented nuclei was counted and expressed as a percentage of total insulin-positive cells.

### *Detection of necrosis*

The levels of lactate dehydrogenase (LDH) released from the cytosol of the cells undergoing necrosis were measured in the culture media of $\beta TC_3$ cultured in absence and presence of calcitriol (1000 nM) by the Clinical Core Laboratory of the Hospital.

### *Northern blot analysis*

Insulin gene expression in $\beta TC_3$ cells, human insulinoma cells, and human islets was analyzed after 48 h of calcitriol exposure by Northern blotting as previously described (37). Relative expression levels of insulin and 18S rRNA were determined by densitometric analysis. Total RNA extracted from $1.5 \times 10^3$ isolated islets and $5 \times 10^5$ insulinoma cells was submitted to the same protocol described for $\beta TC_3$ cells.

### *Insulin release and content*

Basal insulin release was measured in $\beta TC_3$ cells, human insulinoma cells, and human islets. The $\beta TC_3$ cells were seeded at a density of $3 \times 10^4$ cells per well onto 96-multiwell cultures plates and cultured in RPMI 1640 medium in absence and presence of calcitriol (10, 100, and 1000 nM) for 48 h. On the third day, the old medium was aspirated off and cells were washed twice with PBS before being incubated for 1 h in plain RPMI 1640 medium (11 mM glucose). After 1 h, the medium was collected and stored at −20 C, and the cells were harvested and extracted for the measurement of total protein and insulin content. Experiments of insulin release by primary cultures of human insulinoma cells were performed on cultures derived from $1 \times 10^4$ cells seeded onto 96-well tissue culture plates. For insulin secretory studies with isolated human islets, 25 islets were handpicked and plated in 96 multiwell culture plates. Human islets and human insulinoma cells were submitted to the same experimental culture and insulin secretory protocols previously described for $\beta TC_3$ cells. Insulin content of media and cell extracts were measured by RIA (INSIK-5, DiaSorin, Inc., Saluggia, Italy) using rat or human insulin standards as appropriate and were normalized to total protein content. Insulin release and content of cells exposed to calcitriol were then expressed as percentage of control (vehicle-treated) cells.

### *$ERK_1$ and $ERK_2$ phosphorylation*

Phosphorylation of ERK proteins was analyzed in $\beta TC_3$ cells and human islets. After 48 h of culture in standard medium, $\beta TC_3$ cells or human islets were serum starved for 3 h and then alternatively exposed for 20 min to plain medium (without FCS, plus 0.2% albumin), medium plus 10% FCS, or plain medium supplemented with calcitriol (10, 100, and 1,000 nM) in the presence or absence of the MAPK kinase (MEK) inhibitor (UO126 at 2 nM) (38) or the protein kinase C (PKC) inhibitor (staurosporine at 5 nM). After incubation, $\beta TC_3$ cells and islets were resuspended in lysis buffer (30 mM Tris HCl, 5 mM EGTA, 5 mM EDTA, 250 mM sucrose, 1% Triton X-100, 1 mM sodium fluoride, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin). After 1 h at 4 C, lysates were centrifuged at 13,000 rpm for 5 min, and the extracted proteins were analyzed by immunoblotting with antisera against anti-P-$ERK_{1/2}$ and anti-$ERK_{1/2}$ (antimouse and antigoat 1:1,000, respectively, Santa Cruz Biotechnology, Santa Cruz, CA).

### *PKC activity*

After 5 min of exposure to 100 nM calcitriol, the $\beta TC_3$ cells were harvested and extracted in lysis buffer (HEPES-Na 50 mM, Na Cl 250 mM, $MgCl_2$ 5 mM Triton 1%, glycerol 10%, phenylmethylsulfonyl fluoride 10 μg/ml, leupeptin 10 μg/ml, and aprotinin 10 μg/ml). PKC activity in cell lysates was measured by using PepTag assay for nonradioactive detection of PKC (Promega Corp., Madison, WI). This method uses a fluorescent peptide substrate that, when phosphorylated by PKC, alters its net ionic charge from +1 to −1. Phosphorylated and nonphosphorylated substrates are rapidly separated on a agarose gel and quantification of the phosphorylated peptide is performed by spectrofluorimetry.

### *Caspase-3 activity*

The activity of the protease caspase-3 was measured in $\beta TC_3$ cells cultured at a density of $25 \times 10^4$ cells per well onto 12-well culture plates. Cells were exposed for 48 h to calcitriol (10, 100, and 1000 nM) in absence or presence of 50 μM caspase inhibitor Z-VAD-FMK. The $\beta TC_3$ cells were then harvested and caspase-3 activity in whole-cell lysates was determined by using the CaspACE assay system (Promega Corp.) following the manufacturer's instructions. This assay provides a colorimetric substrate (Ac-DEVD) labeled with the chromophore p-nitroaniline (p-NA), which is released from the substrate on cleavage by caspase-3. Free p-nitroaniline produces a yellow color that is measured by a photometer at 405 nm. The amount of yellow color produced is proportional to the amount of DEVDase activity present in the sample.

### *Animal studies*

All animal experiments described in this article were conducted in accord with accepted standards of humane animal care. The *in vivo* antitumorigenic activity of calcitriol was tested in RIP1Tag2 mice at the age of 8–9 wk. A group of mice (n = 6) was treated with calcitriol, given by gastric gavage at the dose of 5 μg/kg body weight every other day for 3 wk. A second group (n = 7) was treated with vehicle only. Mice were monitored twice weekly for body weight and random (from 0800 to 0900 h) blood glucose levels. Because of the development of severe hypoglycemia (blood glucose < 50 mg/dl), 35% glucose was added to the drinking water, starting from the second week of treatment. On the day they were killed, the mice were injected with BrdU (Sigma, 100 mg/kg body weight ip) 6 h before being killed with an overdose of sodium amobarbital. Before death blood samples were drawn for measurement of insulin and calcium levels.

### *Pancreas histology and morphometric analysis of islet and insulinoma volumes*

Pancreases of RIP1Tag2 mice treated with calcitriol or vehicle were retrieved and fixed overnight in Bouin's solution. The following day the pancreases were extensively washed under tap water and were then immersed in 10% buffered formalin until embedded in paraffin. Islets and insulinomas were identified by immunostaining 3- to 5-μm-thick sections for insulin (anti-pig insulin, I8510, Sigma). The volumes of the islets and insulinomas were measured on insulin-stained sections by using a computerized image analysis system (Quinn 2, Leica Corp., Wetzlar, Germany). The volumes of normal islets (longest diameter < 300 μm), hyperplastic islets (longest diameter > 300 and < 600 μm), insulinomas (all insulin-positive masses with longest diameter > 600 μm), and total β-cells (all insulin-positive cells) were measured individually. The number of β-cells undergoing replication or apoptosis was measured in insulinomas by counting the cells stained positively for BrdU and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end- labeling (TUNEL), respectively. Immunostaining with anti-BrdU antibodies (cell proliferation kit, RPN20, Amersham Pharmacia Biotech, Buckinghamshire, UK) and by TUNEL was performed as previously described (39), and number of positive cells was expressed as the percentage of total counted β-cells.

### *Statistical analysis*

*In vitro* studies consisted of a minimum of three independent experiments, each carried out at least in duplicate. The experiments performed on human insulinoma tissue (n = 1) were performed at least in triplicate. Data were expressed as means ± SE. Statistical analysis was performed by using the unpaired *t* test for pairwise comparisons or one- or two-way ANOVA (Tukey *post hoc* test), as appropriate. Statistical significance was considered at $P < 0.05$.

## Results

### *Calcitriol inhibits βTC$_3$ cell growth*

Culture in the presence of calcitriol induced a dose-dependent decrease in the number of βTC$_3$ cells harvested from the culture plates. At 72 h, the number of βTC$_3$ cells cultured in the presence of calcitriol was suppressed to approximately 75%, 65%, and 50% of control at 10, 100, and 1000 nM calcitriol, respectively (Fig. 1A). To determine whether other cell types, known to express the vitamin D receptor, were also sensitive to the antiproliferative effect mediated by calcitriol, rat pituitary GH$_3$, and mouse glucagonoma αTC$_1$ cells were cultured for 48 and 72 h in the presence of calcitriol. Calcitriol decreased the number of αTC$_1$ cells harvested from the dishes to the same extent of βTC$_3$ cells, whereas it was ineffective on GH$_3$ cell growth (not shown).

As shown in Fig. 1C, cell cycle analysis demonstrated an accumulation of βTC$_3$ cells in the G$_0$/G$_1$ phase after exposure to calcitriol, which was already maximally effective at the lowest tested concentration (10 nM). The accumulation in the G$_0$/G$_1$ phase was associated to a concomitant decrease of βTC$_3$ cells in the S and G$_2$/M phases. As expected, calcitriol was ineffective on the cell cycle of pituitary GH$_3$ cells (not shown).

### *Calcitriol decreases number of viable βTC$_3$ cells and induces apoptosis*

As assayed by the MTT assay, culture in the presence of calcitriol reduced the number of viable βTC$_3$ cells in a dose-dependent fashion. After 72 h, viable βTC$_3$ cells were reduced to 75%, 60%, and 50% of control in the presence of 10, 100, and 1000 nM calcitriol, respectively (Fig. 1B). Of note, the number of viable cells declined at the same rate as the number of total cells (Fig. 1A). The number of viable cells decreased similarly in αTC$_1$ exposed to the same calcitriol concentrations but not in GH$_3$ cells, which remained 95% viable even when cultured in 1000 nM calcitriol (not shown).

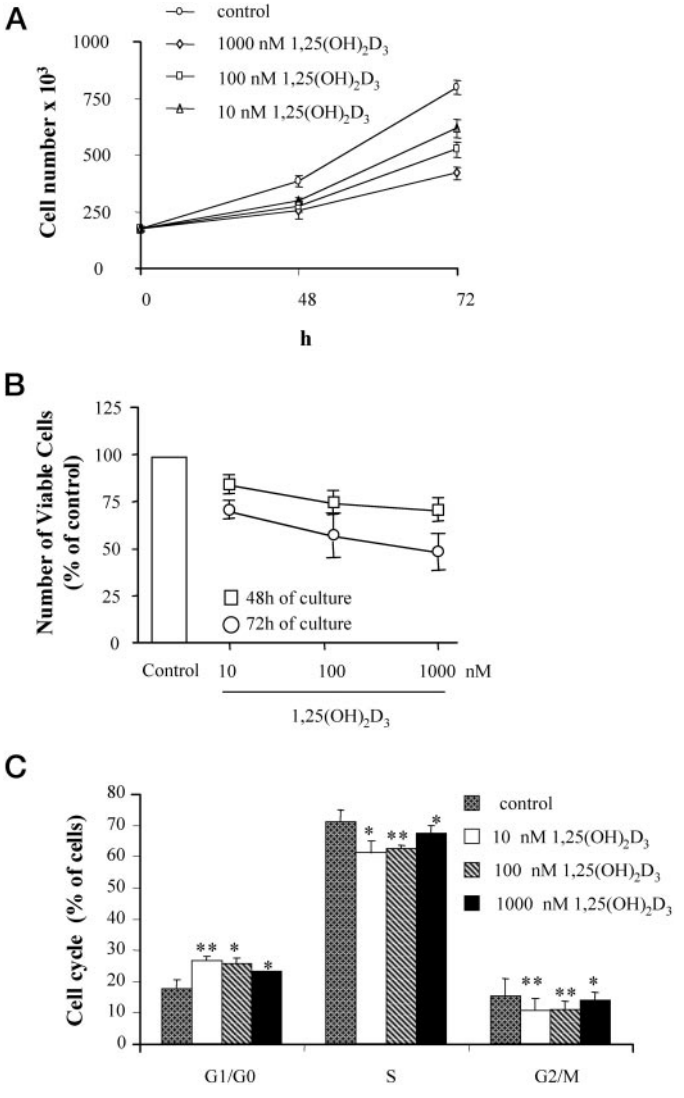


FIG. 1. Effect of calcitriol on βTC$_3$ growth, cell cycle, and number of viable cells. After 48 and 72 h of exposure, calcitriol dose dependently inhibited cell growth (A) and induced a reduction of number of viable cells (B). C, Forty-eight hours of calcitriol administration (10, 100, and 1000 nM) increased the number of βTC$_3$ cells in the G$_0$/G$_1$ phase and concomitantly decreased those in the S and G$_2$/M phases. Data are means ± SE. *, $P < 0.05$ vs. control; **, $P < 0.02$ vs. control (one-way ANOVA, *post hoc* Tukey test); n = 4 experiments, each performed in quadruplicate (A and B); n = 3 experiments, each performed in triplicate (C).

DNA extracted from βTC$_3$ cells cultured in the presence of calcitriol showed the laddered, fragmented pattern typical of apoptosis (Fig. 2A, *upper panel).* The percentage of fragmented DNA increased in a dose-dependent fashion from 19% to 33% and 39% after culture in the presence of 10, 100, and 1000 nM calcitriol, respectively (Fig. 2A, *lower panel).* To determine whether necrosis was contributing to calcitriol-induced βTC$_3$ cell death, LDH levels were measured in the

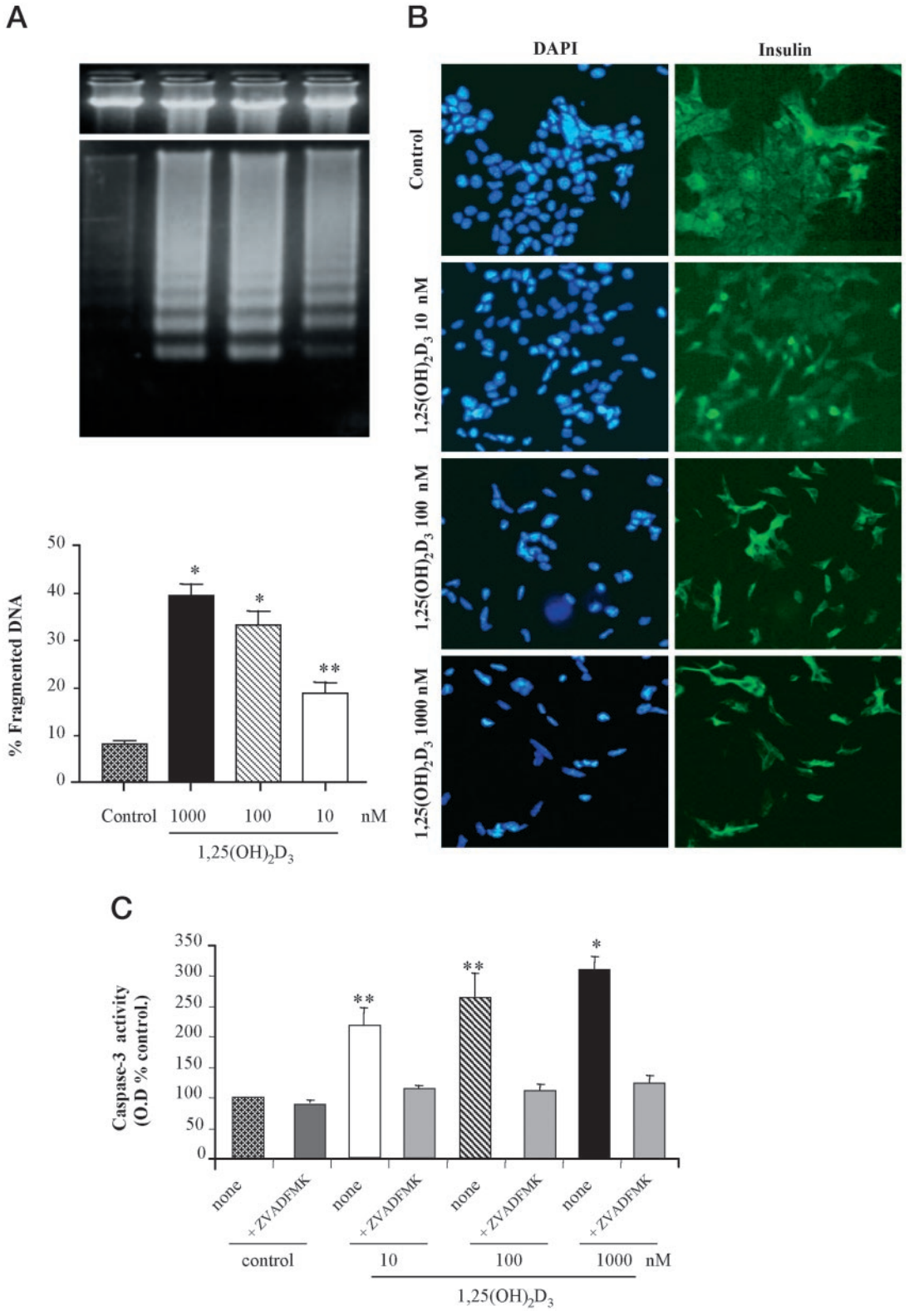


FIG. 2. Calcitriol-induced apoptosis in $\beta TC_3$ cells. A, Exposure to calcitriol induced $\beta TC_3$ cell apoptosis, as shown by the typical ladder-like DNA appearance (*top*). Quantification of fragmented DNA showed a dose-dependent effect of calcitriol (*bottom;* means ± SE, n = 3 experiments, each performed in triplicate; *, $P < 0.01$ *vs.* control; **, $P < 0.05$ *vs.* control and $P < 0.01$ *vs.* 100 and 1000 nM, one-way ANOVA, *post hoc* Tukey test). B, Immunofluorescence for insulin *(green)* and DAPI *(blue)* showing a dose-dependent reduction in $\beta TC_3$ cell number and nuclear condensation and fragmentation of calcitriol-treated cells. C, Apoptosis was associated with a dose-dependent increase in caspase-3 activity, which was prevented by 50 μM caspase-3 inhibitor Z-VAD-FMK (means ± SE, n = 4 experiments, each performed in duplicate; *, $P < 0.02$; **, $P < 0.01$ *vs.* control one-way ANOVA, *post hoc* Tukey test).

culture media. The similar LDH levels detected in the medium containing calcitriol and control medium excluded this possibility (not shown). Furthermore, calcitriol-induced apoptosis was confirmed by immunofluorescence staining with insulin and DAPI performed in $\beta TC_3$ cells exposed or not to calcitriol (Fig. 2B). Nuclear staining with DAPI confirmed the dose-dependent decrease in the number of $\beta TC_3$ cells exposed to calcitriol. Also, the number of condensed and fragmented nuclei, a morphologic feature typical of apoptosis, increased after calcitriol exposure. To identify the cellular pathways involved in calcitriol-induced $\beta TC_3$ apoptosis, caspase-3 activity was measured after 48 and 72 h of exposure to increasing calcitriol concentrations and was compared with the activity of control cells. At 48 h, calcitriol induced an increase in caspase-3 activity to 216%, 263%, and 310% of control cells at 10, 100, and 1000 nM, respectively (Fig. 2C). Calcitriol-induced increase in caspase-3 activity was selectively abolished by the caspase inhibitor (Z-VAD-FMK) (Fig. 2C). Caspase activity remained slightly, albeit significantly, higher than in control cells, even after 72 h (not shown).

### *Calcitriol induces $ERK_1$ and $ERK_2$ phosphorylation via a nongenomic pathway*

It has been previously shown that calcitriol can induce a rapid activation of the MAPK pathway in certain cell types (40) and that, in wild-type p53-retaining cells (such as $\beta TC_3$), inhibition of MAPK efficiently abrogates p53-induced cell death (41). Thus, we explored whether calcitriol could activate ERK1/2 in $\beta TC_3$ cells and ERK inhibition was capable to prevent in the proapoptotic effect of calcitriol on these cells. To do so, proteins extracted from $\beta TC_3$ cells that had been exposed for 20 min to increasing calcitriol concentrations were analyzed by immunoblotting with antisera against unphosphorylated and phosphorylated $ERK_1$ and $ERK_2$. Calcitriol induced an increase in phosphorylated $ERK_1/ERK_2$ to 136%, 150%, 159%, and 184% of control cells after treatment with 1, 10, 100, and 1000 nM calcitriol, respectively (Fig. 3A). Calcitriol-induced ERK phosphorylation was significantly prevented by the MEK inhibitor UO126 and, to a lesser extent, the PKC inhibitor staurosporine (Fig. 3B), which were both ineffective alone (Fig. 3A).

### *Inhibition of MEK and PKC partially prevents calcitriol-induced decrease in number of viable $\beta TC_3$ cells*

UO126 and staurosporine not only prevented calcitriol-induced $ERK_{1/2}$ phosphorylation but also reduced calcitriol-mediated decrease in the number of viable $\beta TC_3$ cells. At the concentration of 2 nM, UO126 increased the number of viable $\beta TC_3$ cells exposed to 1000 nM calcitriol from 68% to 90% of control cells (Fig. 3C). At 5 nM, UO126 lost its protective effect and became cytotoxic (not shown). Calcitriol-induced cell death was also reduced significantly by 5 nM staurosporine that, albeit less potent than UO126, increased the number of viable $\beta TC_3$ cells exposed to 1000 nM calcitriol from 70% to about 82% of control (Fig. 3D). Noteworthy, calcitriol-mediated $\beta TC_3$ cell death was unaffected by forskolin (1–10 μM), wortmannin (100 nM), and *N*-methylarginine (1 μM) (not shown). Calcitriol induced a significant increase in PKC activity of $\beta TC_3$ exposed for 5 min to 100 nM hormone concentration (to 164% of control cells). Calcitriol-induced PKC activation was completely prevented by coadministration of staurosporine (5 nM), which was ineffective when administered alone (Fig. 3E).

### *Calcitriol decreases insulin gene expression, insulin content, and release in $\beta TC_3$ cells*

Insulin gene expression, insulin content, and basal insulin release were measured in $\beta TC_3$ cells cultured in the presence of increasing calcitriol concentrations. These experiments were carried out to determine whether, besides its antitumorigenic activity, calcitriol has also antiinsuligenic effects on mouse insulinoma cells. Indeed, calcitriol dose dependently inhibited insulin mRNA levels in $\beta TC_3$. After 48 h, insulin mRNA levels decreased to 75%, 70%, and 46% of control cells in the presence of 10, 100, and 1000 nM calcitriol, respectively (Fig. 4A). Down-regulation of insulin gene expression was completely prevented by staurosporine and, to a lesser extent, by the phosphatidylinositol 3- kinase inhibitor wortmannin and forskolin. In contrast, UO126 and *N*-methylarginine were ineffective in this regard (Fig. 4B). All tested inhibitors did not change the insulin mRNA levels when administered alone (Fig. 4C). Reduced insulin gene transcription was associated with a significant decrease in $\beta TC_3$ insulin content (Fig. 4D) and release (Fig. 4E).

### *Calcitriol decreases insulin gene expression and insulin secretion but fails to induce apoptosis in primary cultures of benign human insulinoma cells*

Calcitriol also exerted a clear antiinsulinogenic effect on primary cultures derived from a human insulinoma. Insulin mRNA levels of human insulinoma cells exposed to 1000 nM calcitriol decreased to 70% of control (Fig. 5A). Unstimulated insulin release by human insulinoma cells cultured in the presence of 100 and 1000 nM calcitriol also decreased significantly to 75% of control cells (Fig. 5B). The MTT and DNA fragmentation assays were not performed on primary culture of human insulinoma because they were composed also of non-β-cells. Insulinoma cells, identified by insulin and PDX-1 staining, were indeed only about 30–40% of total cell population (Fig. 5C). Because of that, detection of human insulinoma cells undergoing apoptosis was performed by immunocytochemistry of fixed monolayers stained for insulin and DAPI (Fig. 5D). Human insulinoma cells were insensitive to calcitriol-induced cytotoxicity as indicated by the extremely low number of insulin- positive cells showing nuclear condensation or fragmentation (approximately 0.5–1% in either vehicle and calcitriol-cultured cultures).

### *Calcitriol induces $ERK_1$ and $ERK_2$ phosphorylation in human pancreatic islets and decreases insulin gene expression, insulin content, and release*

Aimed at determining whether nontumorigenic human β cells were also sensitive to nongenomic calcitriol effects, islets were exposed for 20 min to calcitriol, and levels of unphosphorylated and phosphorylated $ERK_{1/2}$ were determined by immunoblotting. As shown in Fig. 6A, calcitriol induced a significant increase in phosphorylated $ERK_{1/2}$ to about 120%, 135%, and 140% of control cells in the presence of 10, 100, and 1000 nM calcitriol, respectively. As in $\beta TC_3$ and

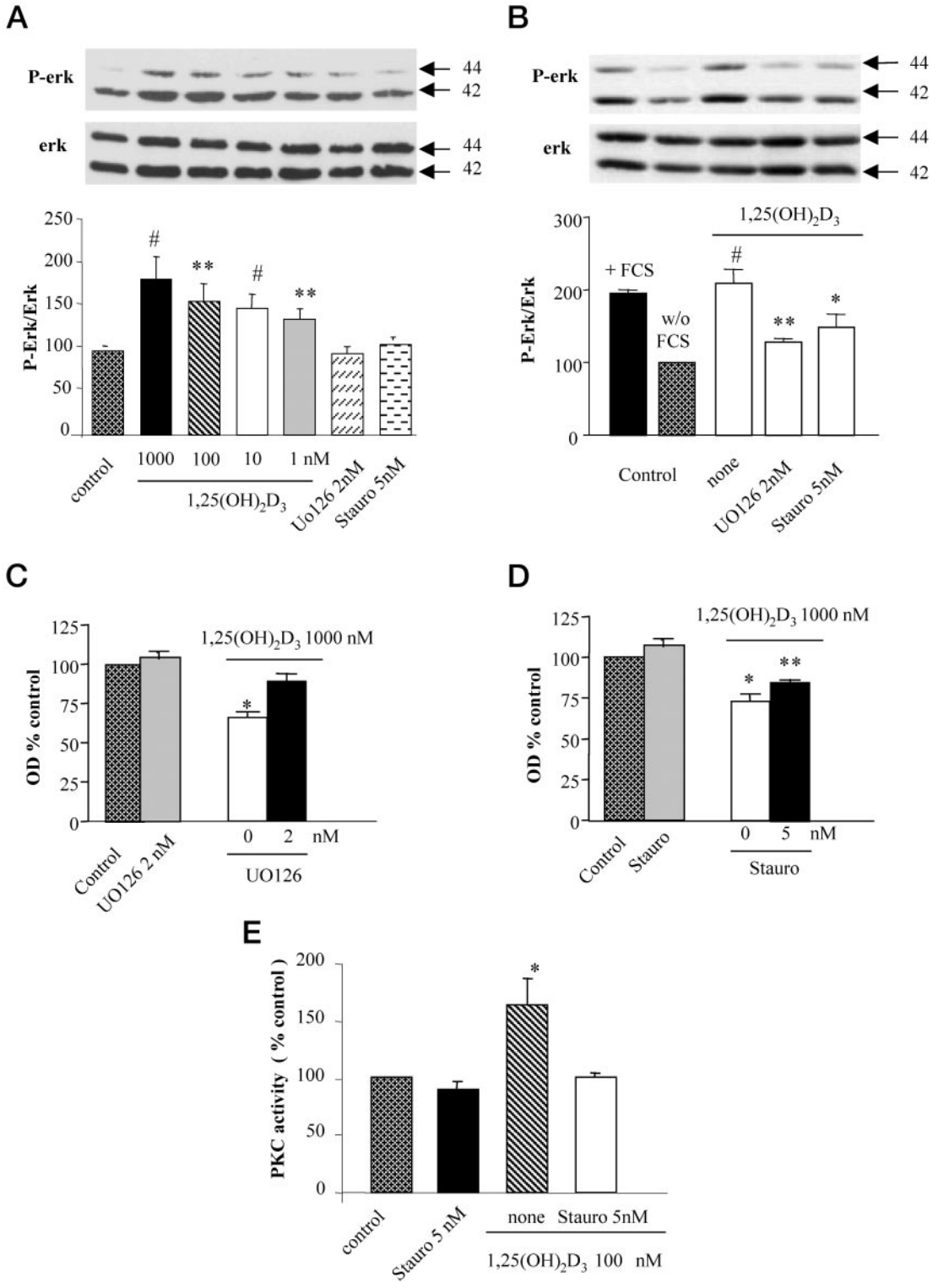


FIG. 3. Calcitriol signal transduction pathway in $\beta TC_3$ cells. A, Calcitriol induced a rapid phosphorylation (within 20 min) of $ERK_1$ (42 kDa) and $ERK_2$ (44 kDa) in a dose-dependent fashion. B, This effect, quantitatively similar to that induced by serum alone, was prevented by UO126 (MEK inhibitor) and staurosporine (PKC inhibitor), which had no effect when administered alone (A) (means ± SE, n = 4 experiments, each performed in triplicate, one-way ANOVA, *post hoc* Tukey test; #, $P < 0.01$ *vs.* control without FCS; **, $P < 0.01$ *vs.* calcitriol alone; *, $P < 0.05$ *vs.* calcitriol alone). C and D, Calcitriol-induced reduction in number of viable cells (assayed by the MTT method) was prevented by UO126 and, to a lesser extent, staurosporine, which were both ineffective when given alone. (means ± SE, n = 4 experiments, each performed in 16 replicates, one-way ANOVA, *post hoc* Tukey test, *, $P < 0.01$ *vs.* control; **, $P < 0.01$ *vs.* control; $P < 0.05$ *vs.* calcitriol alone). E, After 5 min of exposure, calcitriol induced a significant increase in PKC activity that was completely abolished by staurosporine (5 nM), which was ineffective when administered alone (means ± SE, n = 3 experiments, each performed triplicate, one-way ANOVA, *post hoc* Tukey test; *, $P < 0.01$ *vs.* control).

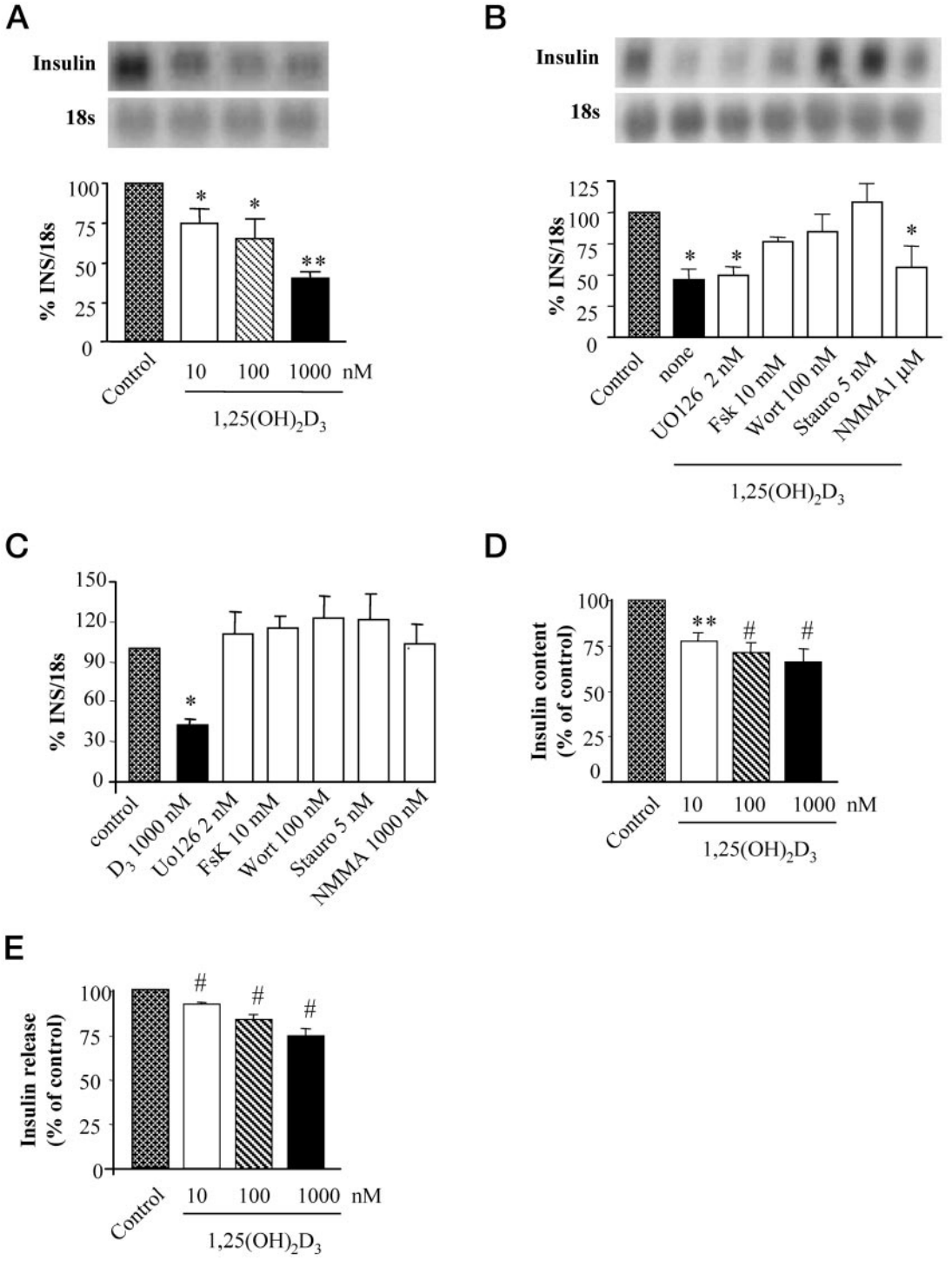


FIG. 4. Effect of calcitriol on insulin gene expression, insulin content, and release in $\beta TC_3$ cells. A, Calcitriol induced a dose-dependent decrease in insulin gene expression (means ± SE, n = 4 experiments, each performed in duplicate, one-way ANOVA, *post hoc* Tukey test; *, $P < 0.01$ *vs.* control; **, $P < 0.01$ *vs.* 10 nmol/liter). B, Down-regulation of insulin gene expression was prevented by staurosporine, wortmannin, and forskolin but not by UO126 and *N*-methylarginine. C, All of these inhibitors were ineffective when administered alone (means ± SE, n = 3 experiments, each performed in duplicate, one-way ANOVA, *post hoc* Tukey test; *, $P < 0.01$ *vs.* control). D and E, Calcitriol significantly decreased content and release of insulin in $\beta TC_3$ cells (means ± SE, n = 7 experiments, each performed in triplicate, one-way ANOVA, *post hoc* Tukey test; **, $P < 0.03$ *vs.* control; #, $P < 0.01$ *vs.* control).

human insulinoma cells, calcitriol induced a significant decrease in mRNA levels, content, and release of insulin in human islets despite the lack of a clear dose-dependent effect (Fig. 6, B–D). Immunofluorescence experiments showed no relevant differences in nuclear morphology of human islet cells cultured in absence or presence of 10 and 1000 nM

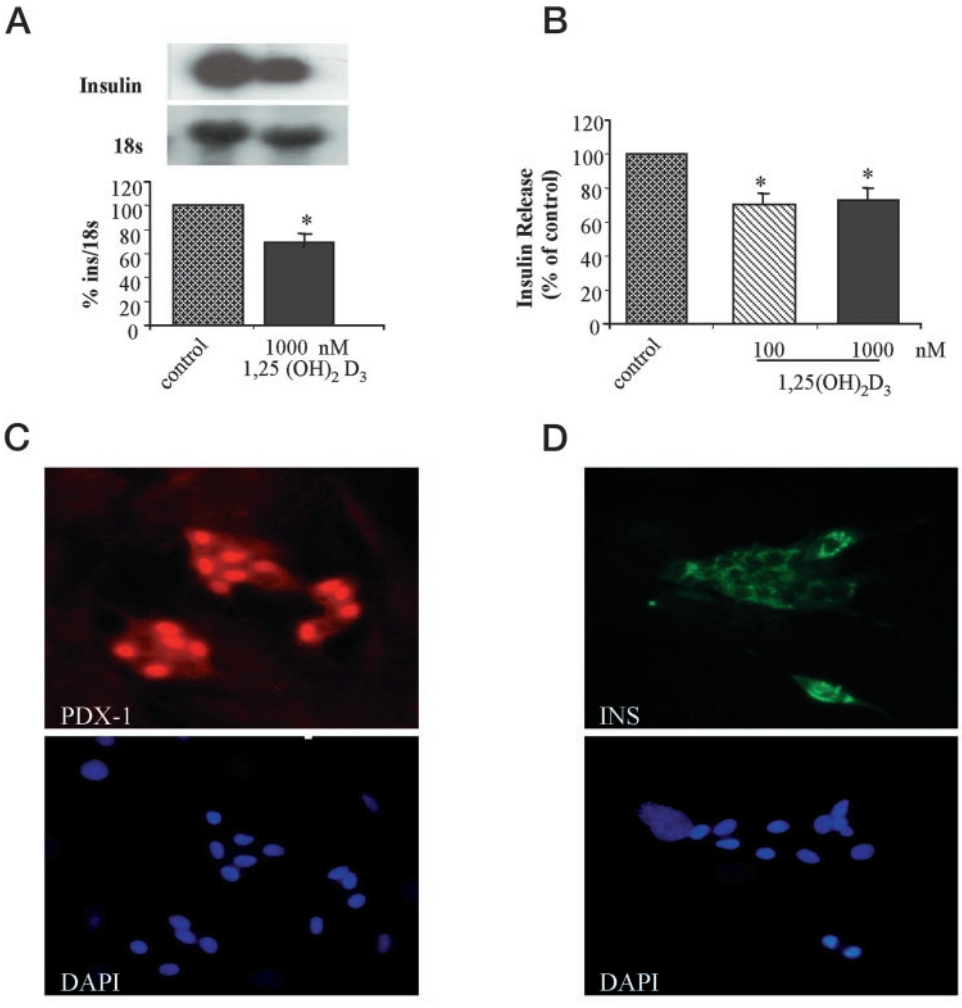


FIG. 5. Effect of calcitriol on primary cultures of a human benign insulinoma. A, Calcitriol (1000 nM) induced a decrease of insulin mRNA levels in benign human insulinoma cells to approximately 70% of vehicle-treated cells (n = 1 experiment performed in triplicate; *, $P < 0.05$ *vs.* control). B, Basal release of insulin by human insulinoma cells was significantly decreased by calcitriol, with 100 nM being already maximally effective (means ± SE, n = 3, each performed in triplicate, one-way ANOVA, *post hoc* Tukey test; *, $P < 0.05$ *vs.* control). C, Immunofluorescence for PDX-1 (*upper panel*) and DAPI (*lower panel*) showing that insulinoma PDX-1-positive cells accounted for only 30–40% of the total cell population. D, Immunofluorescence for insulin (*upper panel*) and DAPI (*lower panel*) did not show morphological evidence of nuclear condensation suggestive of ongoing apoptosis in human insulinoma cells. Two insulin-positive cells undergoing replication are evident on the *bottom right*.

calcitriol, but insulin immunoreactivity was weaker in human islets exposed to calcitriol (data not shown). Together these data demonstrate that the antiinsulinogenic activity of calcitriol is not limited to the neoplastic phenotype but is present also in normal β-cells.

### *Calcitriol treatment reduces the volume of solid β-cell tumors by increasing apoptotic cell death of adenomatous cells*

Insulinoma-bearing RIP1Tag2 mice were treated for 3 wk with calcitriol to ascertain whether the antitumorigenic activity detected *in vitro* was confirmed *in vivo*. At the end of treatment, the number of insulinomas present in the pancreases harvested from calcitriol-treated mice was lower than that of mice treated with vehicle, even though the difference did not reach statistical significance (6.3 ± 1.3 *vs.* 9.2 ± 1.6, respectively, NS). Moreover, morphometric analysis of pancreatic sections stained for insulin showed that treatment with calcitriol did not affect the volume of the normal islet population (Fig. 7A). The volume of the hyperplastic islets decreased after calcitriol administration but not significantly (Fig. 7A). In contrast, the effects of calcitriol were particularly remarkable when considering the global β-cell population and the insulinomas, whose volumes were significantly reduced by calcitriol (Fig. 7B). Reduction of insulinoma volumes was a consequence of increased adenoma cell death rather than reduced replication. As shown in Fig. 7C, about 5% of insulinoma cells of calcitriol-treated mice were undergoing apoptosis, a 3-fold increase, compared with the apoptotic rate detected in the insulinomas of vehicle-treated mice. In contrast, cell replication remained similar in the insulinomas of the two groups ($BrdU^+$ cells 14.3% ± 2.1% *vs.* 13.4% ± 1.6% in calcitriol- *vs.* vehicle-treated, respectively).

Despite the significant reduction in insulinoma volumes, treatment with calcitriol did not affect the glycemic levels of RIP1Tag2 mice. As expected, placebo-treated mice gained weight during the treatment (from 22.8 ± 1.2 to 25.8 ± 1.3 g, $P < 0.01$), but calcitriol-treated mice lost weight (from 22.4 ± 1.3 to 19.8 ± 1.1 g, $P < 0.05$). Such loss of body weight was likely consequent to the anorexia induced by hypercalcemia that, at this dosage of calcitriol, is usually observed in mice (9.4 ± 0.1 *vs.* 10.6 ± 0.3 mg/dl, vehicle *vs.* calcitriol-treated mice, respectively, $P < 0.01$). To determine whether calcitriol had *in vivo* antiinsulinogenic activity, circulating insulin levels were measured in RIP1Tag2 mice before and after treatment with calcitriol or vehicle. Results showed a significant reduction in insulin levels of calcitriol-treated mice (from 33 ± 6 to 14 ± 2 μU/ml; $P < 0.05$), but insulinemia of vehicle-treated mice remained stable in the 30 μU/ml range.

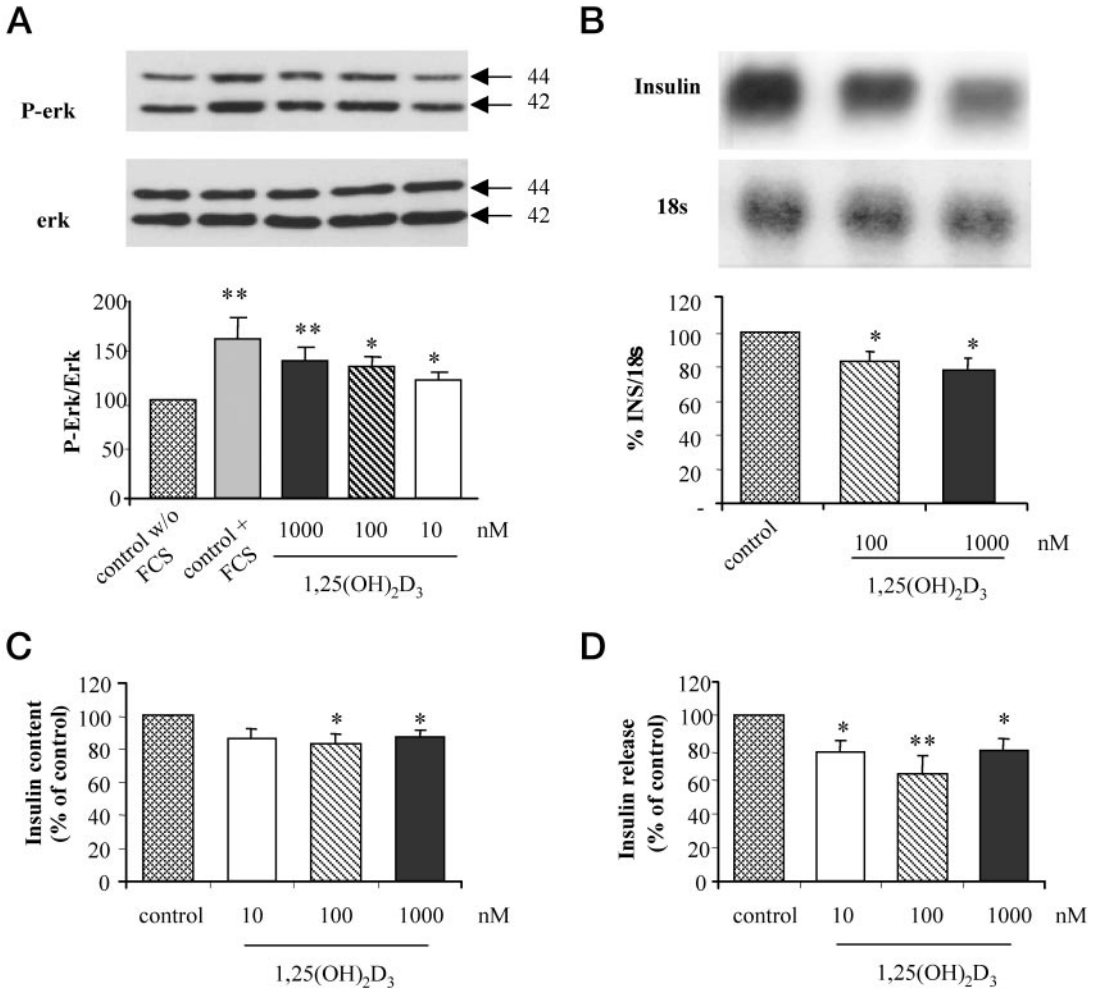


FIG. 6. Effect of calcitriol on human pancreatic islets. A, After 20 min of exposure, calcitriol induced a dose-dependent phosphorylation of ERK 1/2. (means ± SE, n = 3 experiments, each performed in triplicate, one-way ANOVA, *post hoc* Tukey test; *, $P < 0.05$ *vs.* control, **, $P < 0.01$ *vs.* control). B–D, In human islets calcitriol significantly reduced gene expression, content, and release of insulin (means ± SE, n = 4 experiments, each performed in triplicate, one-way ANOVA, *post hoc* Tukey test; *, $P < 0.05$ *vs.* control; ** $P < 0.01$ *vs.* control).

## Discussion

Within the last two decades, a number of studies have shown that the VDR is present not only in classical target tissue such as bone, kidney, and intestine but also in several other nonclassical tissues, including the pancreatic β-cell. Those studies demonstrated that calcitriol and analogs are involved in a multitude of cellular processes including potent antiproliferative and prodifferentiating effects on cancer cell lines *in vitro* and marked antitumoral effects *in vivo* (42). However, no studies have been conducted to address the efficacy of calcitriol in the treatment of β-cell tumors. In this study, we analyzed *in vitro* and *in vivo* the effects of a prolonged exposure to calcitriol on the proliferation, viability, and insulinogenic properties of mouse tumorigenic β-cells and β-cell tumors. We also explored the effects exerted by calcitriol on primary cultures of a human benign insulinoma and isolated human islets.

The results showed that calcitriol induces a wide spectrum of negative effects on mouse insulinoma cells ranging from induction of growth arrest and apoptosis, down-regulation of insulin gene transcription, and reduction of solid β-cell tumors. Calcitriol also induced growth inhibition and apoptosis of $\alpha TC_1$ glucagonoma cells but not pituitary adenoma $GH_3$ cells, which also express the VDR (not shown) (43). The antiproliferative effects that calcitriol induced *in vitro* on $\beta TC_3$ cells were due to a combination of decreased replication and increased cell death. Calcitriol induced an increase in the number of $\beta TC_3$ cells in the $G_0/G_1$ phase of the cell cycle and a concomitant decrease of cells population in the S and $G_2/M$ phases. This effect of calcitriol, previously described to occur in human prostate cancer cells (44) and in colonic adenocarcinoma CaCo-2 cells (45), was already maximal at the concentration of 10 nM in $\beta TC_3$ cells.

Another mechanism by which calcitriol inhibited $\beta TC_3$ growth was the induction of apoptosis. Apoptosis was associated with an increase in caspase-3 activity, indicating the involvement of general apoptotic pathways. Experiments are currently ongoing to explore the mechanisms responsible for the induction of $\beta TC_3$ cell apoptosis by calcitriol. In particular, it will be interesting to determine the role exerted by Bcl-2, which blocks either Apaf-1 activation (46) and p53-induced cell death (47) and whether Bcl-2 overexpression

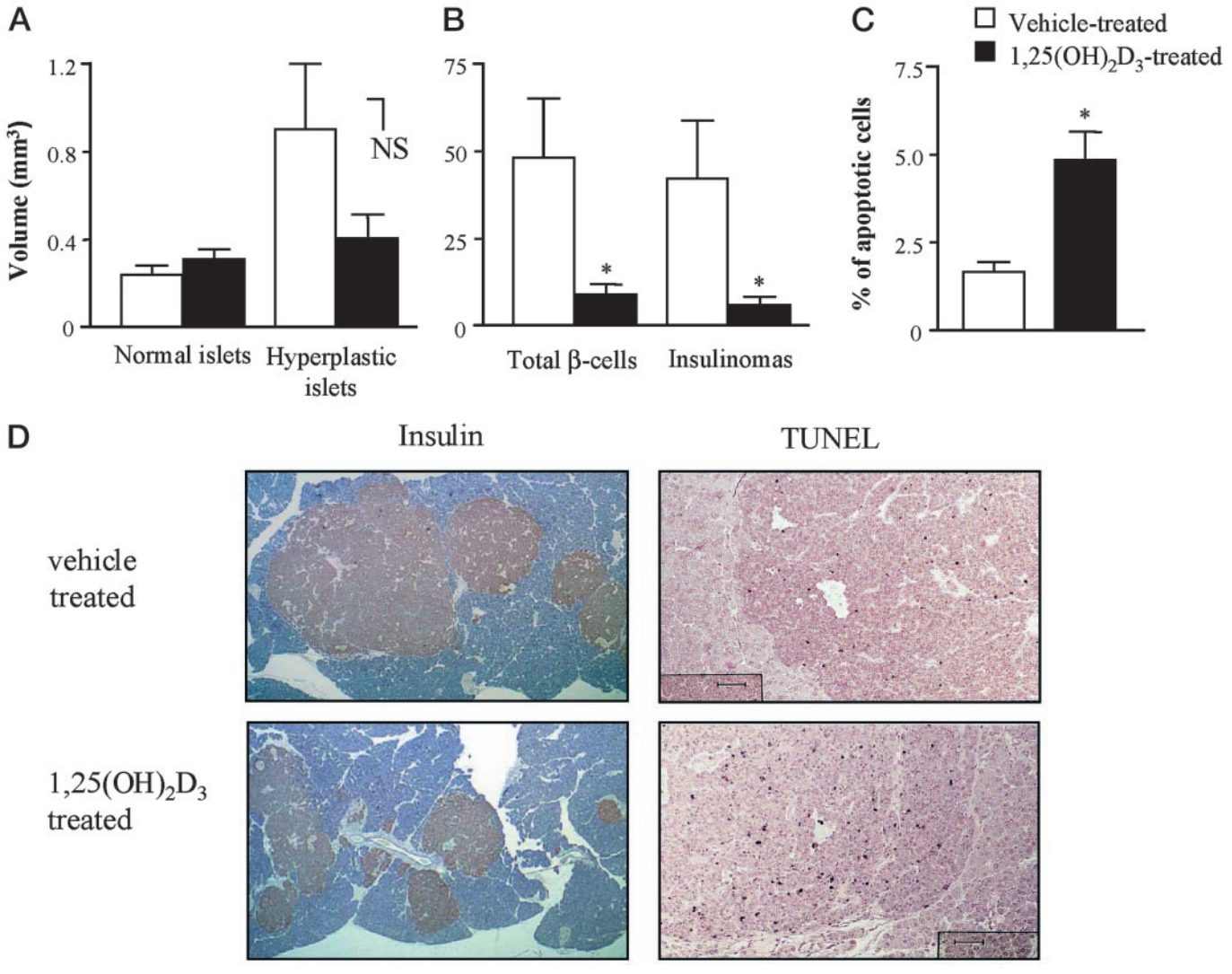


FIG. 7. Calcitriol treatment exerts *in vivo* antineoplastic effect on solid β-cell tumors. A, RIP1Tag2 mice were treated with calcitriol (5 μg/kg orally 3 times/wk, n = 6) or vehicle (n = 7). Morphometric analysis of the harvested pancreas showed no difference in the volumes of the normal and hyperplastic islet populations between calcitriol- and vehicle-treated mice. B, In contrast, a significant reduction of insulinomas and total β-cell mass volumes are evident in calcitriol-treated mice (means ± SE, *, $P < 0.02$ *vs.* vehicle treated). C, A significant increase (~3-fold) in the percentage of cells undergoing apoptosis ($TUNEL^+$) was detected in the insulinomas of calcitriol-treated mice (means ± SE, *, $P < 0.05$ *vs.* calcitriol-treated mice). D, Representative sections of pancreases harvested from vehicle (*upper panels*) and calcitriol-treated (*bottom panels*) mice stained for insulin and TUNEL, showing the reduced insulinoma volumes and the increased number of apoptotic cells rate present in the latter group. Treatment with calcitriol did not induce *in vivo* a decrease in the rate of replication of insulinoma cells.

might prevent βTC3 cells apoptosis, as previously shown in prostate cancer cells exposed to a vitamin $D_3$ analog (46).

Calcitriol may generate biological responses via regulation of gene transcription but also via nongenomic pathways. Indeed, calcitriol increases the intracellular level of many second messengers including cAMP, inositols, calcium, and ceramides that, in turn, activate a variety of protein kinases, including PKC, Raf, MAPK, and Src kinases (48–51). Furthermore, Raf 1 and MEK, upstream effectors of MAPK, are stimulated by calcitriol-induced PKC activation (52). We here provide evidence that, in pancreatic β-cells, calcitriol activates the MAPK cascade via a nongenomic pathway. This occurs in mouse insulinoma cells as well as in human islets as shown by the rapid phosphorylation of $ERK_1$ and $ERK_2$ on calcitriol treatment. $ERK_{1/2}$ phosphorylation was efficaciously prevented by UO126 and staurosporine, indicating that activation of PKC is upstream of MAPK activation. Noteworthy, MAPK activation appears to contribute to calcitriol-induced cytotoxicity because MEK inhibition with UO126 partially prevented this effect in $\beta TC_3$ cells.

Rather unexpectedly, calcitriol decreased insulin gene expression, insulin content, and insulin release of all the β-cell types analyzed in this study, including malignant cells ($\beta TC_3$), benign cells (human insulinoma), and nontumorigenic cells (isolated islets). These data contrast with the vast majority of the studies on the effects of vitamin D on the endocrine pancreas, which consistently found a beneficial effect of calcitriol on β-cell function. It should be noted, however, that almost all of these studies were performed in situations of vitamin D depletion or islet cell damage. In

contrast to the antitumorigenic, the antiinsulinogenic activity exerted by calcitriol did not involve the MAPK pathway. Indeed, down-regulation of insulin gene transcription was not prevented by UO126 but was completely abolished by staurosporine, thereby indicating that this particular effect of calcitriol involves PKC but is independent from MAPK activation. The evidence that in $\beta TC_3$ cells, UO126 efficaciously prevented calcitriol-induced cytotoxicity but failed to reduce the down-regulation of the insulin gene, confirms that the latter phenomenon was specific and not the result of an admixture of viable and dying cells. That the antitumorigenic and the antiinsulinogenic effects exerted by calcitriol on $\beta TC_3$ cells are mediated by distinct intracellular pathways is further supported by the evidence that wortmannin and forskolin significantly reduced the down-regulation of the insulin gene but were completely ineffective in the prevention of calcitriol-induced cytotoxicity. It is now clear that the insulin receptor signaling pathway is active in pancreatic β-cells (53) and plays an important role in β-cell homeostasis (54–58). Interestingly, staurosporine, which prevents PKC-dependent activation of insulin receptor substrate-1 (59), and wortmannin, which inhibits phosphatidylinositol 3-kinase, were both effective in preventing calcitriol-induced down-regulation of insulin mRNA. Perhaps calcitriol may induce the overexpression of insulin receptor substrate-1, which, in pancreatic β-cells, determines reduced mRNA levels, biosynthesis, and content of insulin (60, 61).

Antitumorigenic activity of calcitriol was analyzed *in vivo* in transgenic RIP1Tag2 mice, which represent an extremely well-characterized model of β-cell tumorigenesis. Three weeks of calcitriol treatment significantly altered β-cell tumorigenesis in these mice. Although the volumes of the normal islet populations were similar in calcitriol- and vehicle-treated mice, those of the insulinomas were significantly reduced in the former group. This evidence is consistent with the results obtained *in vitro*, which showed that calcitriol cytotoxicity is limited to the β-cells that show a malignant phenotype. Noteworthy, the decrease in insulinoma volumes was associated to a 3-fold increase in apoptotic cell death, but adenoma cell replication remained similar in the two groups of mice. Other mechanisms, however, may have contributed to the *in vivo* antitumorigenic effect of calcitriol. Besides growth inhibition, calcitriol and analogs have been reported to exert their antitumorigenic activity via inhibition of angiogenesis (62) and by decreasing the invasiveness of cancer cells (63). Perhaps calcitriol may act at different sites of the multistep tumorigenic process described in RIP1Tag2 mice. Inhibition of angiogenesis may reduce *in vivo* the number of islets undergoing the angiogenic switch and the following transition to adenomas, but overexpression of certain adhesion molecules may hinder the transition from adenomas to carcinomas. Despite the reduced insulinoma volumes, glycemic levels of calcitriol- and vehicle-treated mice remained similar. This apparent paradox may depend on the anorexia resulted from calcitriol-induced hypercalcemia. To compensate for hypoglycemia, vehicle-treated mice became hyperphagic and gained weight; instead, calcitriol-treated mice became anorectic and lost weight. Thus, the maintenance of similar blood glucose levels, despite a completely different feeding behavior, may be considered a remarkable clinical achievement. Moreover, circulating insulin levels decreased significantly in calcitriol-treated mice but remained stable in vehicle-treated mice.

In conclusion, we found that calcitriol exerts a potent antitumorigenic and antiinsulinogenic effect on actively replicating murine insulinoma β-cells *in vitro* as well as *in vivo*. At the doses used in this study, calcitriol shows also a clear antiinsulinogenic effect on human β-cells from either normal islets or benign insulinoma. These data provide the rationale for testing the efficacy of calcitriol in the clinical management of patients with β-cell tumors. Calcitriol might be particularly helpful in patients with malignant insulinomas who die of metastatic cancer dissemination and untreatable hypoglycemia in which calcitriol might exert antineoplastic and antiinsulinogenic effects. The availability of calcitriol analogs (64), characterized by a lower calcemic effect, is particularly appealing because they will probably consent to increase the dose of drug that can be safely given to the patient, thereby increasing its biological effect on the neoplastic β-cells.

## Acknowledgments

Received February 20, 2002. Accepted June 13, 2002.

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This work was supported by a postdoctoral fellowship grant from the Ministry of Health of Italy (projects RF98.52 and RF98.50 to F.G., L.P., and M.C.) and Telethon-Italy Grant A118 (to A.M.D.) and Grant E739 (to F.B.F.).

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