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Theophylline-induced Apoptosis is Paralleled by Protein Kinase A-dependent Tissue Transglutaminase Activation in Cancer Cells[1]

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It has been reported that theophylline induces growth inhibition and apoptosis in tumour cells. We report that theophylline induces growth inhibition and apoptosis of several human epithelial tumour cells with an IC:50 of 2.5 mM after 48 h of exposure. Moreover, 2.5 mM theophylline induces the accumulation of cancer cells in S-phase of the cell cycle with a concomitant reduction in the percentage of tumour cells in G_1/G_0 phase. These effects are paralleled by cytoskeletal remodelling with a consequent redistribution of actin fibers and shape change as demonstrated by fluorescence microscopy. The apoptotic death of tumour cells occcurs together with an increase in the expression and activity of the pro-apoptotic enzyme tissue transglutaminase (tTGase). All these effects are promptly antagonized by the specific PKA inhibitor KT5720, suggesting the involvement of cAMP intracellular elevation and, consequently, PKA activation. On the other hand, growth inhibition and tTGase expression and activity are potentiated by retinoic acid, a tTGase inducer. Therefore, a mechanistic model of theophylline action and anti-tumour strategies based on the concomitant use of theophylline and agents that potentiate tTGase activity can be hypothesized.

Key words: apoptosis, protein kinase A, retinoic acid, theophylline, tissue transglutaminase.

Methylxanthines are natural compounds of interest because of the widespread ingestion of methylxanthine-containing beverages such as coffee and tea. In addition, some methylxanthines have been used as therapeutic agents. Among these alkaloids, the best known are theophylline (1,3-dimethyl-xanthine), theobromine (3,7-dimethylxanthine), and caffeine (1,3,7-trimethylxanthine) (*1*). It has been shown that these compounds act as phosphodiesterase inhibitors, thus elevating intracellular cyclic AMP (cAMP) levels (*2*). Theophylline can also act as an adenosine antagonist on neutrophils (*3*). Previous observations have indicated that methylxanthines may have an antitumorigenic action (*4–6*). In addition, theophylline has been shown to induce differentiation in melanoma cells and to reduce the invasion of highly metastatic melanoma cells (*7*). It has, moreover, been reported by us and others that theophylline is also able to induce cell growth inhibition, at least in part, through the induction of apoptotic cell death (*8, 9*). Theophylline-induced apoptosis can be related in part to both intracellular cAMP elevation due to phosphodiesterase inhibition and antagonism against adenosine A2A receptors (*9, 10*), even if theophylline-induced apoptosis unrelated to phosphodiesterase inhibition has been also described (*11*). We have previously reported that theophylline enhances the expression and activity of tissue transglutaminase (tTGase) in B16 murine melanoma cells, and the induction of tTGase activity is inversely correlated to the invasivity and metastatic potential of tumour cells (*12*).

Transglutaminases are calcium-dependent enzymes that catalyze post-translational modifications of proteins by the formation of covalent crosslinks between γ-carboxamido groups of glutamine endoresidues and ε-amino groups of lysine endoresidues (*13*). Tissue transglutaminase (tTGase; type II TGase) is a ubiquitous member of the transglutaminase enzyme family. This protein, however, is expressed at very high levels in endothelial cells and condrocytes and is localized mainly at the cytoplasmic level (*14*).

Growth factors (*i.e.* epidermal growth factor, EGF and transforming growth factor β, TGFβ) and cytokines (*15*) can regulate tTGase gene expression in cancer cells. Previous data have shown that tTGase activity is directly involved in some cellular activities, *e.g.*, receptor mediated endocytosis, programmed cell death or apoptosis, cell adhe-

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Abbreviations: cAMP, cyclic AMP; db-cAMP, dibutyryl-cAMP; DMC, *N,N*-dimethylated casein; EGF, epidermal growth factor; FITC, fluorescein isothiocyanate; NP40, nonidet P40; PBS, phosphate buffered saline; PI, propidium iodide; PKA, protein kinase A; RA, retinoic acid; SDS-PAGE, sodium-dodecylsulphate polyacrylamide gel electrophoresis; Spd, spermidine; tTGase, tissue transglutaminase; TGFβ, transforming growth factor β

sion, cell growth, and tumour cell proliferation (*16*). Moreover, several studies have shown that tTGase gene expression is regulated by cAMP and retinoids (*17, 18*).

It has been known for more than 50 years that retinoids are potent agents for the control of cellular differentiation and proliferation. Several studies have shown that retinoids can suppress the process of tumorigenesis both in vitro and *in vivo* (*19–21*), and that cells exposed to retinoic acid (RA) arrest in the G1 phase of the cell cycle. However, the molecular mechanism underlying this arrest remains unclear. It has been reported that the down regulation of cyclin D3 expression is critical for the differentiation of F9 teratocarcinoma cells induced by RA (*22*). It was also demonstrated that RA can induce differentiation together with tTGase increased activity in HL-60 human myeloid leukemia cells, and that this effect is potentiated by the stimulation of protein kinase A (PKA) by dibutyryl-cAMP (db-cAMP) (*23*).

In this study we have evaluated the effects of theophylline on growth inhibition, cell cycle distribution, apoptosis and cell remodelling of several epithelial cancer cells. Moreover, we have studied whether the effects induced by theophylline can be suppressed by the specific PKA inhibitor KT5720. Finally, we evaluated whether tTGase is involved in the apoptosis induced by theophylline. We have studied the activity and expression of the pro-apoptotic enzyme tTGase following treatment with theophylline and/or KT5720, and evaluated whether the potentiation of tTGase activity and expression caused by RA can also enhance the growth inhibition induced by theophylline.

MATERIALS AND METHODS

Cell Culture and Cell Proliferation Assays—Human rhynopharyngeal KB and lung H1355 epidermoid carcinoma cell lines, obtained from the American Type Tissue Culture Collection, Rockville, MD, were grown in DMEM supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% L-glutamine, and 1% sodium pyruvate. The human melanoma cell line GLL-19, obtained from the American Type Tissue Culture Collection, Rockville, MD, was grown in RPMI-1640 supplemented with heat inactivated 10% FBS, 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine. The cells were grown in a humidified atmosphere of 95% air/5% CO_2 at 37°C. For cell proliferation experiments, 1.5×10^5 control or theophylline and/or KT5720 and/or RA-treated cells were seeded in 6-multiwell plates and incubated at 37°C, Theophylline and/or KT5720 and/or RA were added 24 h after seeding and every 24 h thereafter. At the selected times the cell number was determined with a haemocytometric counter after the addition of trypan blue dye.

Internucleosomal DNA Fragmentation (Ladder)—DNA fragmentation was measured after the extraction of low molecular weight DNA. Briefly, 10×10^6 cells were resuspended in 900 μl 1× Tris-EDTA buffer and lysed with 25 μl 20% SDS. The DNA was precipitated in ethanol for 6 h in the presence of 5 M NaCl. The high molecular weight fraction was sedimented by high speed centrifugation, and the fragmented DNA was extracted from the aqueous phase with phenol and chloroform and then precipitated with ethanol. After resuspension in water, the DNA was subjected to electrophoresis in 1.5% agarose gels and visualized by ultraviolet light following ethidium bromide staining.

Evaluation of Apoptosis and Cell Cycle by DNA-Flow Cytometry—Cells were centrifuged and stained directly in a propidium iodide (PI) solution (50 μg PI in 0.1% sodium citrate, 0.1% NP40, pH 7.4) overnight at 4°C in the dark. Flow cytometric analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (mod.310) for data analysis. Cell cycle data analysis was performed with the CELL-FIT programme (Becton Dickinson). Pulse area versus pulse width gating was performed to avoid doublets from the G_2/M region. To evaluate cell apoptosis, PI fluorescence was collected as FL2 (Log scale) by the CellFIT software (Becton Dickinson). The data were acquired after analysis of at least 20,000 events in three different experiments. The statistical significance of the difference in cell cycle pattern with and without the addition of theophylline was evaluated by analysis of variance. Statistical evaluation was performed with the BMDP statistical software package.

Immunodetection of tTGase—For tTGase immunodetection, cells growing in complete alpha DME medium (80% confluent) were washed three times with phosphate-buffered saline (PBS). Five micrograms of total homogenate protein was subjected to sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% polyacrylamide gel. The proteins were then electroblotted to type HA 0.45-mm pore nitrocellulose paper (Bio-Rad, Richmond, CA). The primary antibody to TGase (mouse monoclonal antibody; Dako) was diluted 1:2,000 with a blocking solution containing 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% FBS. The secondary antibody, mouse anti-rabbit (Sigma, S. Louis, MO) conjugated with peroxidase was diluted 1:3,000 with the same blocking solution. The intensities of the bands associated with tTGase were determined by laser scanning using a common software (Gel-Pro Analyzer, Media Cybernetics, Silver Spring, MD).

tTGase Assay—Enzyme activity was assayed by measuring the incorporation of [^{3}H]spermidine trihydrochloride (Spd, specific activity 15 Ci/mmol; Amersham, Buckinghamshire, UK) into N,N-Dimethylated casein (DMC). Assay mixtures (100 μl) containing 125 mM Tris-HCl buffer, pH 8.0, 10 mM DTT, 2.5 mM Ca^{2+}, 50 nM [^{3}H]Spd, and 0.2 mg of DMC were incubated at 37°C for 1 h in the presence of various amounts of cellular homogenate. Blanks were simultaneously run with radioactive Spd in the presence of 5 mM EGTA. The reactions were stopped by adding 1.0 ml of 10% trichloroacetic acid containing 2 mM unlabeled spermidine and the samples were centrifuged. The resulting precipitates were washed twice by suspension in the above solution, dissolved in 1.0 ml of 0.1 N NaOH, and finally counted in 5 ml of Pico-Fluor 40 scintillation mixture (Packard).

Cell Cytoskeleton Studies—Cells were plated on 35 × 10 mm tissue culture dishes with DMEM medium for 12 h, fixed, washed two times with PBS containing sodium azide, permeabilized with 0.1% Triton X-100 in PBS, and stained for F-actin with 2 μg/ml fluorescein isothiocyanate (FITC)–conjugated phalloidin (F-PHD; Sigma) after stimulation with theophylline in the presence or absence of KT5720.

Statistical Analysis—All data are expressed as mean ± SD. Statistical analysis was performed by analysis of variance (ANOVA) with Neumann-Keul's multiple comparison

test or Kolmogorov-Smirnov where appropriate.

RESULTS

Effects of Theophylline on Growth Inhibition and Apoptosis of Several Epithelial Cancer Cells—We evaluated whether different concentrations of theophylline can induce growth inhibition of human epidermoid rhynopharingeal KB and lung H1355 cancer cells and melanoma GLL-19 cells. We found that theophylline induces time- and dose-dependent growth inhibition of all three cell lines as evaluated by haemocytometric cell count (Fig. 1A). In detail, exposure of the three cell lines to 2.5 mM theophylline for 48 h induced almost 50% growth inhibition without cell cytotoxicity as evaluated by trypan blue assay and by study of the cell growth profile (Fig. 1A and data not shown). Under the same experimental conditions, theophylline was also able to induce apoptosis as evaluated by FACS analysis after labelling with propidium iodide (Fig. 1, B–G). In fact, 2.5 mM theophylline caused 48, 40, and 49% apoptotic cell death in the KB, H1355, and GLL-19 cell lines, respectively (Fig. 1, B–G). At the same time, theophylline induced a decrease in the percentage of tumour cells in G_1/G_0 phase of the cell cycle with a concomitant accumulation of cells in S-phase (Table I). On the other hand, the addition of 1 mM theophylline induced no significant changes in the distribution of tumour cells in the cell cycle (Table I).

Therefore, theophylline causes cell growth inhibition, apoptosis and significant changes in the cell cycle of epithelial cancer cells.

KT5720 Antagonizes Cell Growth Inhibition and Apoptosis Induced by Theophylline—We have evaluated whether

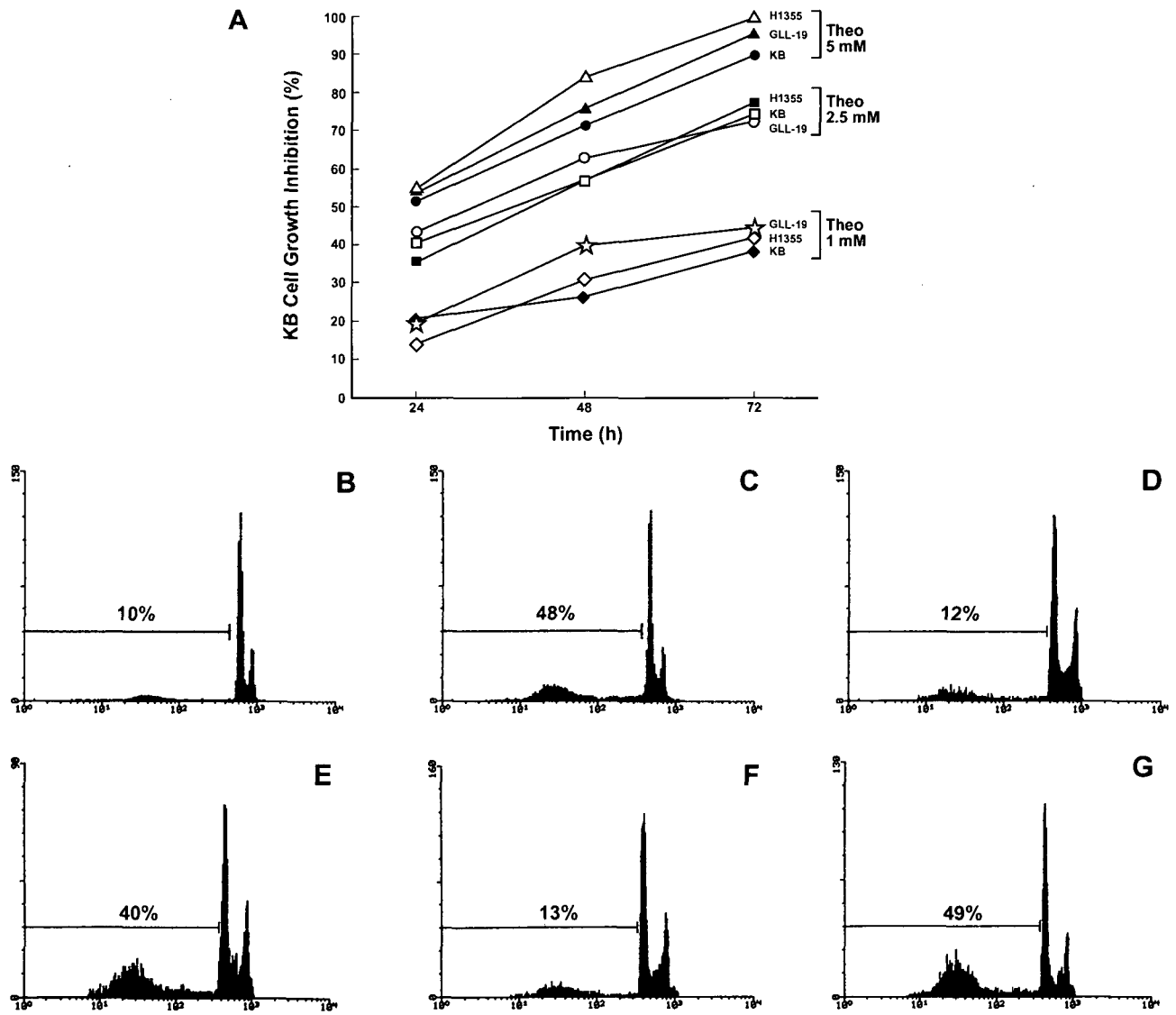

Fig. 1. **Effects of theophylline on growth inhibiton and apoptosis of human epithelial tumour cells.** (A) KB, H1355, and GLL-19 cells were treated with different concentrations of theophylline as described in "MATERIALS AND METHODS." At the selected times, the cells were counted in a haemocytometer. The values are expressed as growth inhibition. SDs were always less than 10%. (B–G) FACS analysis after propidium iodide labelling of KB, H1355, and GLL-19 cells as described in "MATERIALS AND METHODS." Untreated (B) and 2.5 mM theophylline-treated (C) KB cells; untreated (D) and 2.5 mM theophylline-treated (E) H1355 cells; untreated (F) and 2.5 mM theophylline-treated (G) GLL-19 cells. Bars show the apoptotic peaks with the relative % of apoptotic events.

TABLE I. **Effects theophylline exposure for 48 h on tumour cell cycle distribution.**

	% of cells (mean ± SE)		
	G_0/G_1 phase	S phase	G_2/M phase
KB cells			
Untreated	60.30 ± 4.40	30.10 ± 0.87	9.60 ± 1.38
1 mM theophylline	58.80 ± 2.32	27.90 ± 0.52	13.30 ± 0.92
2.5 mM theophylline	17.70 ± 0.15	73.30 ± 4.50	9.00 ± 0.29
GLL-19 cells			
Untreated	58.00 ± 4.32	28.50 ± 1.01	12.50 ± 1.71
1 mM theophylline	61.00 ± 4.01	26.50 ± 1.20	12.50 ± 1.49
2.5 mM theophylline	20.00 ± 1.44	63.70 ± 0.24	16.30 ± 0.74
H1355 cells			
Untreated	58.00 ± 2.22	25.50 ± 1.52	16.50 ± 1.91
1 mM theophylline	61.00 ± 3.01	26.50 ± 1.10	12.50 ± 1.69
2.5 mM theophylline	20.00 ± 1.44	63.70 ± 0.24	16.30 ± 0.74

The percent distribution of tumour cells in the cell cycle was evaluated by FACS analysis after nuclear labelling with propidium iodide. The results are expressed as the mean of the data acquired after analysis of at least 20,000 events in three different experiments performed in triplicate ± standard error (SE).

the inhibition of PKA activation by the specific inhibitor KT5720 is also able to antagonize the effects of theophylline on cell growth inhibition and apoptosis of KB cells. We found that 6 μM KT5720 alone induces time-dependent growth inhibition that reaches 25% after 72 h of exposure, as evaluated by haemocytometric cell count (Fig. 2A). Theophylline again caused time-dependent growth inhibition without apparent cytotoxicity, but when cells were exposed to both agents, strong antagonism was recorded (Fig. 2A). In fact, after 48 and 72 h of treatment with 6 μM KT5720 and 2.5 mM theophylline, only about 20% growth inhibition was recorded (Fig. 2A). Analogous results were obtained when apoptosis was evaluated by gel ladder and FACS analysis. In fact, the exposure of KB cells to 2.5 mM theophylline for 48 h induced apoptosis in 47% of the cell population, but when the cells were treated with the two agents together, strong antagonism was observed since only 20% of cells underwent apoptosis (Fig. 2, B, E, and F, respectively). KT5720 alone caused apoptosis in only 15% of the cell pop-

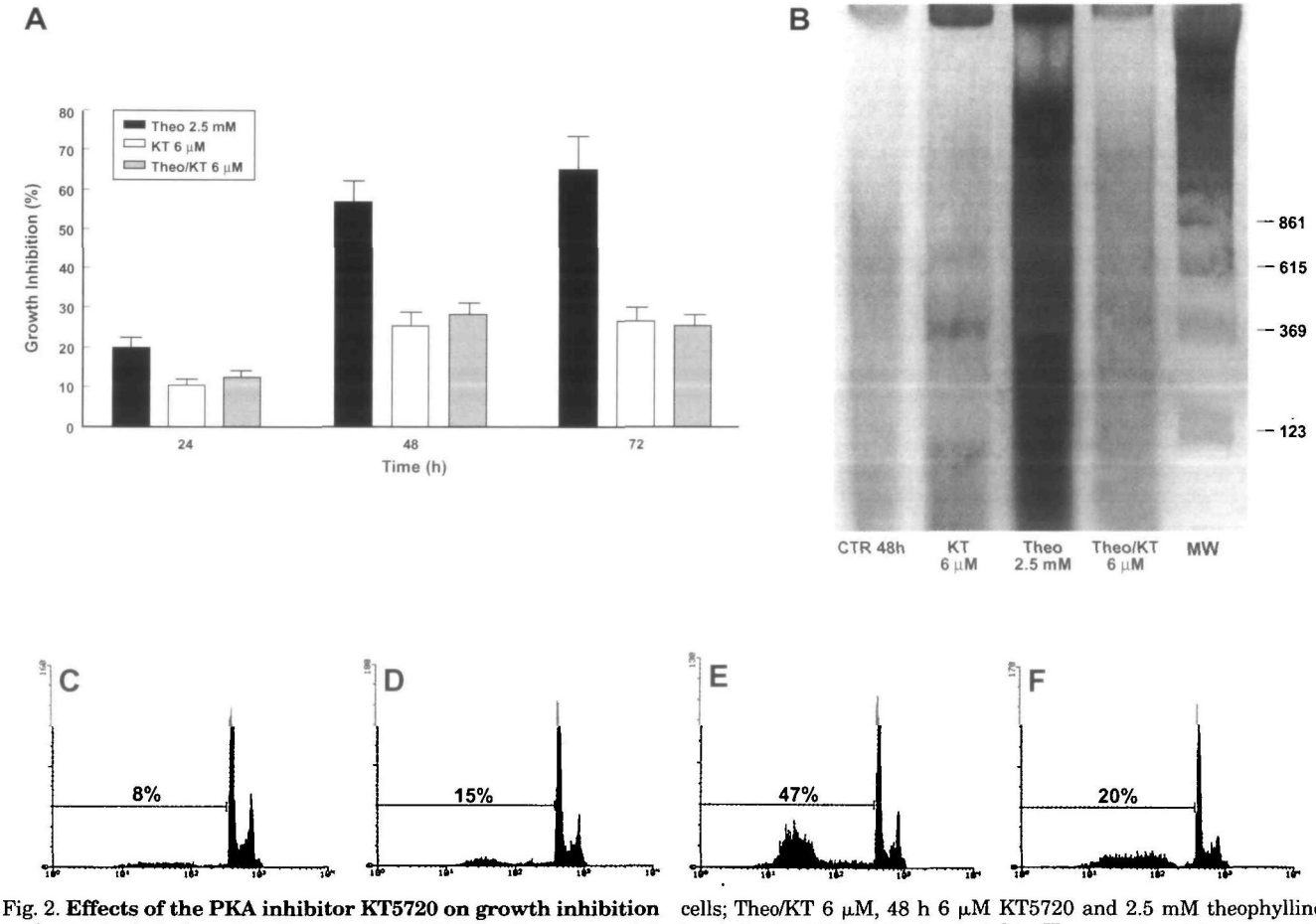

Fig. 2. **Effects of the PKA inhibitor KT5720 on growth inhibition and apoptosis induced by theophylline.** (A) KB cells were seeded and treated every 24 h with 2.5 mM theophylline and/or 6 μM KT5720 as described in "MATERIALS AND METHODS." Cell number was determined with a hamocytometer at the selected times. (B) KB cells were seeded and treated with 2.5 mM theophylline and/or 6 μM KT5720 for 48 h. Then the cells were collected and processed for internucleosomic DNA fragmentation as described in "MATERIALS AND METHODS." CTR 48 h, untreated cells; KT 6 μM, 48 h 6 μM KT5720-treated cells; Theo 2.5 mM, 48 h 2.5 mM theophylline-treated cells; Theo/KT 6 μM, 48 h 6 μM KT5720 and 2.5 mM theophylline-treated cells; MW, molecular weights. The experiment is representative of at least three separate experiments that always gave similar results. (C–F) FACS analysis after propidium iodide labelling of KB cells as described in "MATERIALS AND METHODS." C, untreated cells; D, 48 h 6 μM KT5720-treated cells; E, 48 h 2.5 mM theophylline-treated cells; F, 48 h 6 μM KT5720 and 2.5 mM theophylline-treated cells. Bars show the apoptotic peaks with the relative % of apoptotic events.

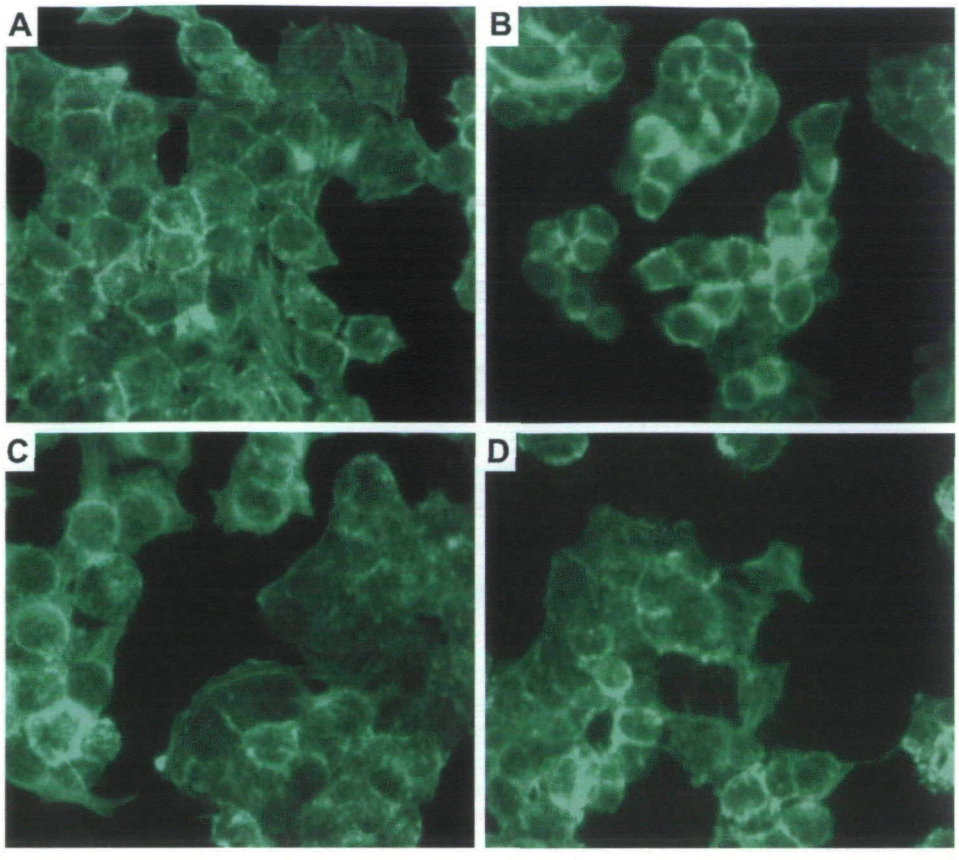

Fig. 3. **Effects of theophylline and KT5720 on intracellular actin fibers distribution.** KB cells were treated with theophylline and/or KT5720, and actin distribution was visualized under a fluorescence microscope as described in "MATERIALS AND METHODS." (A) Untreated KB cells; (B) 48 h 2.5 mM theophylline-treated KB cells; (C) 48 h 6 mM KT5720-treated cells; (D) 48 h 6 μM KT5720 and 2.5 mM theophylline-treated cells. The experiment is representative of at least three different experiments that gave always similar results.

ulation (Fig. 2, B and D). Analogous results were obtained using the PKA inhibitor myristoilated 14-22 amide (data not shown).

Therefore, the selective inhibition of PKA activity by KT5720 is paralleled by antagonism of cell growth inhibition and apoptosis suggesting that the effects of theophylline are mostly due to PKA activation.

Effects of Theophylline and KT5720 on Epidermoid Cancer KB Cell Remodelling—It has been reported that theophylline reduces the invasivity and metastatic potential of melanoma cells, which also depends upon cell motility and cytoskeletal modifications (*7*). Therefore, we evaluated whether theophylline induces cell remodelling under our experimental conditions. We studied actin cytoplasmic distribution by fluorescence microscopy after labelling KB cells with FITC-conjugated phalloidin. Moreover, we again evaluated whether the specific PKA inhibitor, KT5720, can antagonize such effects. The incubation of KB cells with theophylline induced shape changes and modified the normal distribution of actin-containing stress fibers (Fig. 3B). After exposure of KB cells to 2.5 mM theophylline for 48 h at 37°C, the cells showed a rounded shape and disruption of the actin cytoskeleton characterized by the depolymerization of actin fibers, which assumed a peripheral and spotted cytoplasmic distribution that caused cell rounding but preserved attachment (Fig. 3B). Incubation with 6 μM KT5720 did not cause relevant modifications (Fig. 3C), but concomitant exposure of KB cells to both agents prevented the changes in cell shape or cytoskeleton organization induced by theophylline (Fig. 3D). Therefore, theophylline induces cell shape remodelling through actin fiber reorganization likely due to PKA activation.

Effects of Theophylline and KT5720 on tTGase Expression and Activity—Since tTGase is involved in the induction of both apoptosis and cell invasivity, and we have previously shown that theophylline can activate tTGase, we evaluated the effects of theophylline and KT5720 on tTGase expression and activity in KB cells (*16*). We found that exposure of KB cells to 2.5 mM theophylline for 48 h induced an approximately 2-fold increase, while 6 μM KT5720 alone induced about a 70% reduction of tTGase expression (Fig. 4, A and B). Moreover, the concomitant exposure of KB cells to theophylline and KT5720 completely antagonized the effects of theophylline on tTGase expression (Fig. 4, A and B). Analogous results were again obtained using the PKA inhibitor 14-22 amide (data not shown). We evaluated the effects of theophylline on tTGase activity with a kinase assay using dimethylcaseine as substrate. We found that treatment of KB cells with 2.5 mM theophylline for 48 h induced an approximately 2-fold increase in tTGase activity, while 6 μM KT5720 caused about a 50% reduction in enzyme function (Fig. 4C). Again the simultaneous exposure of KB cells to both agents completely antagonized the effects of theophylline (Fig. 4C). In order to demonstrate the involvement of tTGase in the apoptosis induced by theophylline, we treated KB cells with RA, an inducer of tTGase activity. The exposure of KB cells to 100 nM RA or to 1 mM theophylline for 48 h caused an approximately 0.5-fold increase in tTGase expression as evaluated by western blot assay (Fig. 4D). The simultaneous exposure of KB cells to both drugs induced about a 2-fold increase in enzyme expression (Fig. 4D). This effect

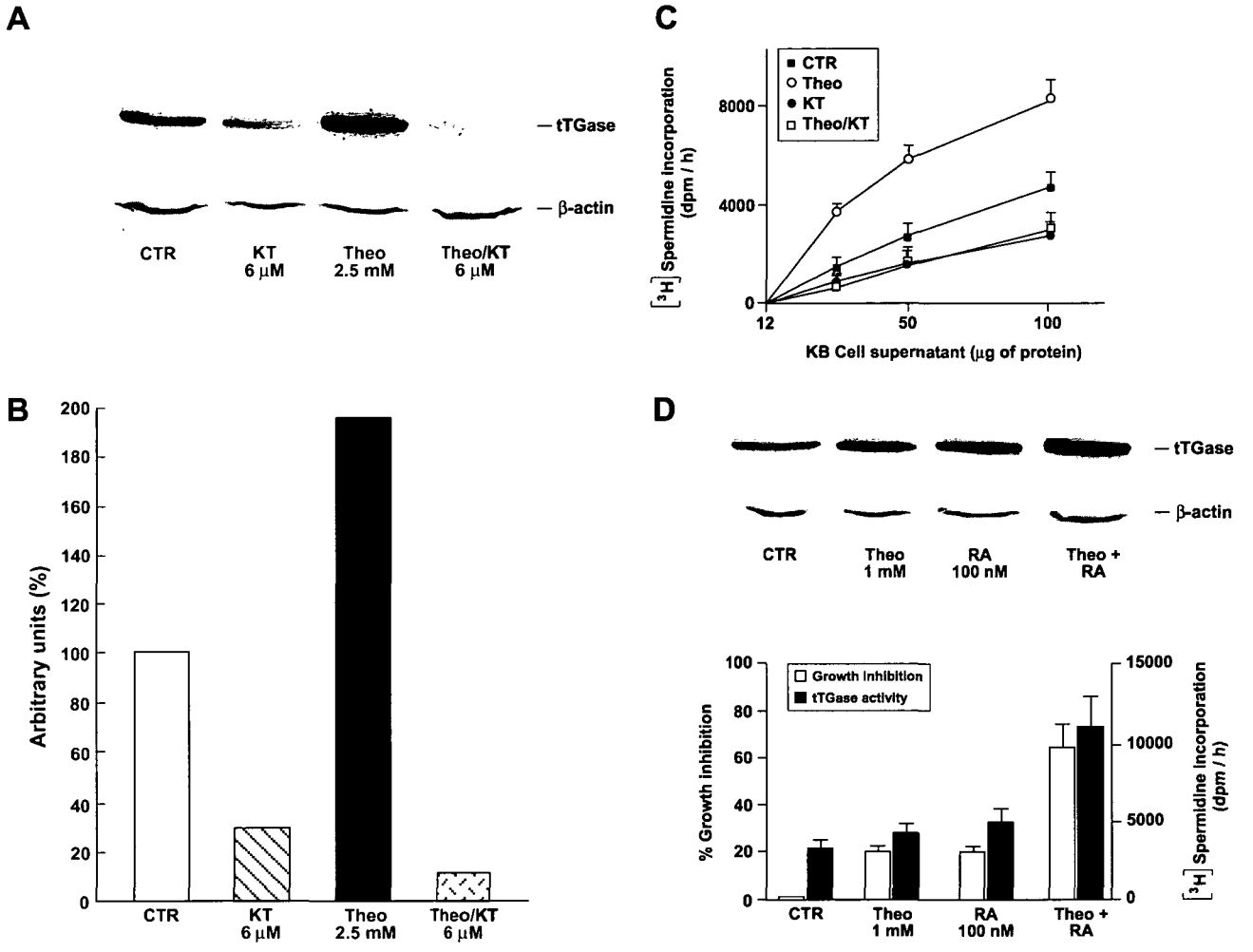

Fig. 4. **Effects of theophylline and KT5720 on the expression and activity of tTGase.** (A) KB cells were seeded and treated with theophylline and/or KT5720. After 48 h of treatment, the cells were processed for the immunodetection of tTGase or β-actin by Western blotting as described in "MATERIALS AND METHODS." CTR, untreated cells; KT 6 μM, 48 h 6 μM KT5720-treated cells; Theo 2.5 mM, 48 h 2.5 mM theophylline-treated cells; Theo/KT 6 μM, 48 h 6 μM KT5720 and 2.5 mM theophylline-treated cells. The experiment is representative of at least three different experiments that always gave similar results. (B) Laser scans of the specific band associated with tTGase. The intensities of the bands are expressed in arbitrary units (%). CTR, untreated cells; KT 6 μM, 48 h 6 μM KT5720-treated cells; Theo 2.5 mM, 48 h 2.5 mM theophylline-treated cells; Theo/KT 6 μM: 48 h 6 μM KT5720 and 2.5 mM theophylline-treated cells. (C) KB cells were cultured for 48 h in the absence or presence of 2.5 mM theophylline and/or 6 μM KT5720. At that time, the cells were processed for the determination of tTGase activity as described in "MATERIALS AND METHODS." Untreated KB cells (■); 2.5 mM theophylline-treated KB cells (○); 6 μM KT5720-treated KB cells (●); 6 μM KT5720 and 2.5 mM theophylline-treated cells (□). The experiment was performed three times with SDs always less than 5%. Bars, SD. (D) KB cells were cultured for 48 h in the absence or presence of 2.5 mM theophylline and/or 100 nM RA. (Upper panel) Cells were processed for the immunodetection of tTGase by Western blotting as described in "MATERIALS AND METHODS." CTR, untreated KB cells; Theo 1 mM, 48 h 1 mM theophylline-treated cells; RA 100 nM, 48 h 100 nM RA-treated cells; Theo + RA, 48 h 1 mM theophylline- and 100 nM RA-treated cells. The experiment is representative of at least three separate experiments that always gave similar results. (Lower panel) Cell growth (□) was evaluated by haemocytometric cell count and tTGase activity (■) was as measured by enzymatic assay as described in MATERIALS AND METHODS. CTR, untreated KB cells; Theo 1 mM, 48 h 1 mM theophylline-treated cells; RA 100 nM, 48 h 100 nM RA-treated cells; Theo + RA, 48 h 1 mM theophylline- and 100 nM RA-treated cells. The experiment was performed three times with SDs always less than 5%. Bars, SD.

was paralleled by an analogous increase in activity. In fact, the single agents induced about a 50% increase in tTGase activity, while the simultaneous exposure of KB cells to both agents produced about a 2-fold increase in enzyme activity as evaluated by enzymatic assay (Fig. 4D). These biochemical effects occurred together with a potentiation of growth inhibition induced by the two drugs. In fact, 100 nM RA and 1 mM theophylline alone for 48 h induced about 20% growth inhibition, while the addition of both agents had an anti-proliferative effect of about 65% without cell cytotoxicity as evaluated by trypan blue assay (Fig. 4D).

These data suggest that theophylline-induced apoptosis is paralleled by an increase in the expression and activity of the pro-apoptotic enzyme tTGase. These effects were again antagonized by the suppression of PKA activity by KT5720 and potentiated by the addition of the tTGase inducer RA.

DISCUSSION

Studies have been already carried out on the effects of methylxanthines on neoplastic cells. In fact, it has been reported that theophylline induces growth inhibition and apoptosis in melanoma and haematologic cells, causing a decrease in bcl-2 expression in eosinophils (*7, 11, 24*). Moreover, theophylline and cisplatin synergize in inducing apoptosis and down-regulating bcl-2 in human granulosa cells transformed by a mutated p53 and Ha-ras oncogene (*8*). The use of theophylline in the treatment of chemo-resistant leukemias has also been suggested based on its ability to reduce the expression of bcl-2 and induce apoptosis in pretreated chronic lymphatic leukemia cells (*25*). These biological effects can be related to the inhibition of phosphodiesterase activity and the consequent increase in intracellular cAMP. In turn, cAMP can both activate PKA and interact with the actin cytoskeleton to determine the structural modifications of the cell in apoptosis (*9*). Another proposed mechanism is theophylline antagonism against A2A cell membrane receptors for adenosine (*10*). Moreover, we have reported that theophylline activates tTGase in murine melanoma B16-F10 cells, and that this effect is paralleled by reduced metastatic potential of the melanoma cells (*12*). In the present study, we evaluated the ability of theophylline to induce cell growth inhibition and apoptosis in human epithelial cancer cells. We found that theophylline concentrations attainable *in vivo* induce growth inhibition without apparent cytotoxicity and apoptosis of human epidermoid cancer KB and H1355 and melanoma GLL-19 cells. Under these experimental conditions, theophylline also induced an accumulation of tumour cells in S-phase of the cell cycle with a concomitant reduction in the number of cells in G_0/G_1 phase. Theophylline-induced growth inhibition and apoptosis were recorded together with structural reorganization of actin microfilaments in the periphery of the cytoplasm. Since tTGase is involved in the triggering and execution of apoptotic cell death, in growth inhibition, and in cytoskeletal organization, we also studied the expression and activity of this enzyme in theophylline-treated cells (*15, 16, 26*), and found both to increase. The increase in tTGase expression and function and the growth inhibition induced by theophylline were potentiated by the addition of RA, another inducer of tTGase. All the biological and biochemical effects induced by theophylline were antagonized by the specific PKA inhibitors KT5720 and the myristoilated amide 14-22, suggesting the involvement of cAMP and PKA in the effects induced by theophylline, even if other mechanisms of action cannot be completely excluded. The synergism between theophylline and RA supports the involvement of tTGase in apoptosis induced by theophylline since the potentiation of anti-proliferative effects was paralleled by enhanced tTGase expression and activity. Moreover, the potentiation of antiproliferative effects is not likely due to cAMP effects on RA receptor expression since it has been reported that cAMP reduce the expression of all three RA receptor mRNAs in murine B16 melanoma cells (*27*), even if the modulation of RA receptor expression was not evaluated in our experimental model. On the other hand, it has already been described that the addition of Triiodothyronine to RA-treated HL-60 cells potentiates the differentiation induced by RA by increasing intracellular cAMP levels (*28*). Moreover, it has been reported that retinoic acid and db-cAMP enhance differentiation and tTGase activity in HL-60 cells (*23*).

On the basis of these considerations, it can be hypothesized that theophylline induces elevated intracellular cAMP levels that activate PKA causing apoptosis by triggering pro-apoptotic mechanisms involving tTGase activation and cytoskeletal reorganization. The use of agents that increase the activity of tTGase in combination with theophylline could be proposed for the treatment of tumours of epithelial origin. However, further experimental work is required in order to understand the biochemical basis and therapeutic potential of such combination.

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