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Sphingosine Kinase Mediates Cyclic AMP Suppression of Apoptosis in Rat Periosteal Cells

MOHAMED MACHWATE, SEVGI B. RODAN, GIDEON A. RODAN, and SHUN-ICHI HARADA

Department of Bone Biology and Osteoporosis Research, Merck Research Laboratories, WP26A-1000, West Point, Pennsylvania 19486

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ABSTRACT

Prostaglandin E stimulates bone formation in humans and animals, and increases intracellular cAMP in osteoblastic cells. We found that cAMP inhibits apoptosis in osteoblastic cells, and examined the mechanism of this effect. We report that the cAMP elevating agent, forskolin, increases cell number in the rat periosteal cell line (RP-11), by suppressing apoptosis in a cell type-specific manner. In RP-11, forskolin transiently up-regulates extracellular signal-regulated kinase activity, a known suppressor of apoptosis. PD98059, a selective inhibitor of the extracellular signal-regulated kinase pathway, only partially reverses the antiapoptotic effect of forskolin, which suggests an additional mechanism for cAMP action. We found that forskolin stimulates cytosolic sphingosine kinase (SPK) activity in these cells; in two other osteoblastic cell lines, however, forskolin does not suppress apoptosis. In contrast to the partial opposing effect of PD98059 to forskolin action, *N*,*N*-dimethylsphingosine, a specific inhibitor of SPK, completely reverses the antiapoptotic effect of forskolin, and has no effect on apoptosis in the absence of forskolin. These findings show for the first time that cAMP activates SPK in a cell-type-specific manner, and suggest that cAMP suppression of apoptosis in RP-11 periosteal cells is mediated by its stimulation of SPK.

Apoptosis plays an important role in development, homeostasis, and the elimination of damaged cells (reviewed in Cohen, 1993; Thompson, 1995). A number of proteins involved in apoptosis have been identified, including the caspases, activated via Fas and TNF receptors (reviewed in Wallach, 1997), and the Bcl2 family members that can either induce or suppress apoptosis (reviewed in Gajewski and Thompson, 1996; Golstein, 1997).

Sphingolipid metabolites (e.g., ceramide, SP, and SPP), have been implicated in signal transduction (reviewed in Spiegel and Merrill, 1996). Sphingoid bases are formed either by *de novo* synthesis or during the turnover of complex sphingolipids metabolized by specific enzymes (reviewed in Spiegel and Merrill, 1996). Recent studies in mesangial cells and Swiss 3T3 fibroblasts (Coroneos *et al.*, 1995; Cuvillier *et al.*, 1996) found that apoptosis-inducing interleukin-1 and TNFα stimulate sphingomyelinase activity, whereas ceramidase and SPK activities are increased by growth factors. Thus, this is one of the signaling pathways that responds differently to certain cytokines (via elevation of ceramide) and growth factors (via SP and SPP). Branching pathways of sphingolipid metabolism may mediate contrasting or opposing effects: ceramide induces apoptosis in several cell types (reviewed in Hannun and Obeid, 1995), whereas SPP suppresses it (Cuvillier *et al.*, 1996). SPP, the SPK product, was originally identified as an intracellular mitogenic messenger (Zhang *et al.*, 1991; Olivera and Spiegel, 1993; Miyake *et al.*, 1995; Wu *et al.*, 1995) and was shown subsequently to be involved in cell motility (Sadahira *et al.*, 1992; Bornfeldt *et al.*, 1995), activation of muscarinic K+ currents in atrial myocytes (Van Koppen *et al.*, 1996), and neurite retraction (Postma *et al.*, 1996). Recently, SPP was reported to suppress apoptosis induced by ceramide and Fas activation in Swiss 3T3 and HL60 cells (Cuvillier *et al.*, 1996). Activation of protein kinase C was shown to induce SPK activity in these cells, which suggests that SPP may be a downstream mediator of suppression of apoptosis (Cuvillier *et al.*, 1996).

cAMP was reported to suppress apoptosis in rat pheochromocytoma PC12 cells (Xia *et al.*, 1995) and in human neutrophils (Rossi *et al.*, 1995), but it induces apoptosis in human mammary carcinoma MCF-7 cells (Boe *et al.*, 1995). In PC12 cells, cAMP up-regulates ERK, and suppresses JNK and p38 activity (Xia *et al.*, 1995). It was proposed that, in these cells, survival is determined by the balance between ERK and JNK/p38 activities. On the other hand, in the human neuroblastoma SHEP cell line, ERK mediates Fas-induced apoptosis (Goillot *et al.*, 1997), and in cerebellar neurons, insulin-like growth factor-1 supports cell survival without activating

ABBREVIATIONS: TNF, tumor necrosis factor; ERK, extracellular signal-regulated kinase; SPK, sphingosine kinase; DMS, *N*,*N*-dimethylsphingosine; SP, sphingosine; SPP, sphingosine-1 phosphate; JNK, c-*Jun* amino-terminal kinase; PDGF, platelet-derived growth factor; Fas-L, Fas ligand; dbcAMP, dibutyryl cAMP; ALP, alkaline phosphatase; OC, osteocalcin; MEK, mitogen extracellular signal-regulated kinase kinase; PG, prostaglandin; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; TUNEL, terminal deoxynucleotidyl transferase dioxygenin-labeled UTP nick-end labeling; NFκB, nuclear factor κB.

ERK. The antiapoptotic effect of insulin-like growth factor-1 in these cells is mediated by the serine-threonine protein kinase Akt/Protein kinase B via phosphatidyl inositol-3 kinase (Dudek *et al.*, 1997). Thus, pathways other than ERK might play a role in regulating apoptosis in response to extracellular stimuli, including elevation of intracellular cAMP.

cAMP is the second messenger of PGE2 and -E1 and, possibly, several other osteogenic factors, such as parathyroid hormone and fluoride. It thus would be of great interest to understand the cellular and molecular mechanisms mediating the effect of cAMP. In the present study, we describe a newly immortalized periosteal cell line (RP-11) in which cAMP increases cell number by suppressing apoptosis. We investigated the intracellular mechanisms involved in this effect, and found that both ERK and SPK activities are induced by cAMP in a cell-type specific manner. The effects of specific inhibitors for these kinases suggest that although ERK is important for the survival of RP-11 cells, SPK may play a specific role in mediating cAMP suppression of apoptosis in these cells.

Experimental Procedures

Materials

SP, SPP, and DMS were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). PD98059, ERK, and JNK activity detection kits are from New England Biolabs (Beverly, MA). Fas ligand (Fas-L)was from Alexis (San Diego, CA). Culture media were from Gibco-BRL (Gaithersburg, MD). FBS was from JRH Biosciences (Lenexa, KS). Random primer DNA-labeling kit (Arlington Heights, IL), [α-^{32}P]deoxy-CTP, [γ-^{32}P]ATP, and [^{3}H]thymidine were from Amersham (Arlington Heights, IL). All other chemicals used are from Sigma Chemical (St. Louis, MO).

Methods

Periosteal cell isolation and immortalization. Periostea were dissected from the anteromedial tibial surface of 2–3-week-old Sprague Dawley rats (Taconic, Germantown, NY), as described previously (Nakahara *et al.*, 1991). Periostea were subjected to collagenase (1 mg/ml) digestion for 1 hr at 37° to release periosteal cells. Primary periosteal cells were cultured in presence of DMEM supplemented with 10% FBS and were subcultured every week. Colonies of cells that formed after 6 weeks of culture were isolated using cloning cylinders and were subcloned by limiting dilution as described previously (Grigoriadis *et al.*, 1996). A clonal periosteal cell line RP-11 was selected and characterized.

Cell culture. RP-11 cells were cultured in DMEM supplemented with 10% FBS. RCT-3 (Heath *et al.*, 1989) and C5.18 (Grigoriadis *et al.*, 1996) are fetal rat calvaria-derived cell lines. RCT-3 osteoblastic cells, immortalized by simian virus 40 large T antigen, were cultured in Ham's F-12 medium containing 5% FBS and 400 μg/ml of Geneticin 418 (GIBCO-BRL, Gaithersburg, MD). C5.18 chondro-osteogenic cells, kindly provided by Dr. Jane E. Aubin (University of Toronto, Toronto, Canada) were cultured in minimal essential medium-α containing 15% FBS. To examine the effect of forskolin and PGE1 on cell number, RP-11, C5.18, and RCT-3 cells, plated at 100,000 cells per well in 24-well plates (Costar, Cambridge, MA), were cultured for two days and were treated with forskolin (10 μM) or PGE1 (1 μM) in presence of 2% serum. After 3 or 6 days, cells were trypsinized and counted using a Coulter counter (Coulter Electronics, Lutton, England).

RNA isolation and Northern blot analysis. RP-11 periosteal cells were cultured until confluence. Total RNA was extracted as described previously (Chomczynski and Sacchi, 1987). RNA (15 μg) was electrophoresed through 1% agarose-formaldehyde and blotted onto a nylon membrane (Hybond N; Amersham). The filters were hybridized at 42C in hybridization buffer containing mouse type I collagen α 1, rat ALP, or rat OC cDNA probes labeled using a random primer DNA-labeling kit and [α-^{32}P]deoxy-CTP.

[^{3}H]-Thymidine incorporation. RP-11, RCT-3 and C5.18 cells were plated in 24-well plates at 50,000 cells per well and cultured for 24 hr. Cells were cultured without serum for an additional 24 hr and were treated with forskolin (10 μM) in the presence of 2% FBS for 20 hr. [^{3}H]thymidine (0.1 μCi/ml) was added 2 hr before culture arrest, and incorporated thymidine was determined as described previously (Rodan *et al.*, 1987).

Detection of apoptosis. RP-11 cells, plated at 50,000 cells/cm^2 in 24-well plates, were cultured for 2 days in DMEM supplemented with 10% FBS. Cells were treated with forskolin (10 μM), Fas-L (200 μg/ml), PD98059 (0.5–5 μM), SPP(0.01–10 μM), or DMS (0.01–10 μM) for 48 or 72 hr in DMEM with 2% FBS. For analysis of DNA content by flow cytometry, the cells were trypsinized and single cell suspensions were prepared as described previously (Rak *et al.*, 1996). Briefly, the cells were fixed in 3:1 (v/v) ethanol/phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, and 1.4 mM KH_2PO_4, pH 7.3), incubated with 0.5 μg/ml RNase A and stained with propidium iodide at a final concentration of 50 μg/ml. DNA content and cell cycle profile were analyzed with a FACScan flow cytometer (Becton Dickinson, San Francisco, CA). The data acquisition and analysis were performed using cellQuest and ModFit software (Becton Dickinson, San Francisco, CA), respectively. The percent protection from apoptosis was calculated as follows: [(Control − Treated) / Control] × 100.

For *in situ* identification of apoptotic cells, the TUNEL assay was used according to the manufacturer's recommendations (Oncor, Gaithersburg, MD). Briefly, RP-11 cells were fixed in 2% paraformaldehyde, permeabilized, and incubated with nucleotide terminal transferase in the presence of digoxygenin-11-dUTP. Labeled cells were identified using an antidigoxygenin HRP-conjugated antibody.

ERK and JNK assays. ERK and JNK activity was determined by immunoprecipitation and *in vitro* kinase assays, according to the manufacturer's recommendations (New England Biolabs). Briefly, ERK and JNK were precipitated from cell lysates using a specific anti-ERK antibody and c-Jun-GST fusion protein, respectively. The precipitates were subjected to *in vitro* kinase assays in the presence of ELK1 and c-*Jun* as substrates for ERK and JNK respectively, and cold ATP. Phosphorylation of ELK1 and c-*Jun* was detected by western blotting using specific antibodies that recognize phosphorylated ELK1 and c-*Jun*.

Sphingosine kinase assay. Cells were plated and cultured in 6-well plates as described above. After 2 days of culture, cells were treated with 10 μM forskolin for 1, 2, and 4 hr, or with dbcAMP (0.1, 1, and 10 μM) for 2 hr in the presence of 2% FBS. The cells were lysed, scraped, and sonicated for 2 min in 20 mM Tris, pH 7.5, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mM Na_3VO_4 and 1 mM NaF at 4°. The sonicate was centrifuged for 60 min at 100,000 × *g*. SPK activity was measured as described previously (Olivera and Spiegel., 1993). Briefly, 200 μl of supernatant was incubated with 50 μM SP, 0.2% BSA, and [γ-^{32}P]ATP (1 mM, 0.2 Ci/mmol) for 30 min at 37°. Lipids were extracted with 240 μl of chloroform and analyzed for SPP content by thin layer chromatography developed in butanol/acetic acid/water (3:1:1), followed by autoradiography.

Results

Forskolin increases cell number by suppressing apoptosis in RP-11 periosteal cells in a cell-type specific manner. RP-11 cells were spontaneously immortalized from primary periosteal cells. As shown in Fig. 1A, after confluence, these cells express mRNA for type I collagen, ALP, and

OC, phenotypic markers for osteoblasts. Treatment with forskolin (10 μM) for 3 and 6 days increased RP-11 cell number by about 2-fold (Fig. 1B) compared with control. Similar effects were obtained with PGE1 (1 μM), a stimulator of adenylate cyclase in these cells (Fig. 1C). This effect was cell type-specific, because in RCT-3 and C5.18 calvaria-derived osteoblastic cells, forskolin decreased cell number (Fig. 1B) and suppressed proliferation measured by [^{3}H]thymidine incorporation (Fig. 1D), which may account for the decrease in cell number. In contrast, forskolin had no effect on [^{3}H]thymidine incorporation in RP-11 cells (Fig. 1D), which suggests that the increase in cell number did not result from increased proliferation.

Quantitative analysis of DNA content by flow cytometry showed that in the presence of 2% FBS, 21% and 35% of RP-11 cells undergo apoptosis after 48 and 72 hr, respectively (Fig. 2A). Forskolin (10 μM) suppressed apoptosis by 72% at 48 hr and by 79% at 72 hr (Fig. 2A). Similar antiapoptotic effects were obtained with PGE1 (1 μM) (Fig. 2A). The DNA content profile showed that forskolin did not affect cell proliferation in RP-11 cells (the percentage of cells in S and G2 phases were 29.27% in control versus 29.32% in the presence of forskolin). In RCT-3 and C5.18 cells, forskolin had no effect on apoptosis (11.5% and 4.9% in controls versus 11.0% and 5.1% in the presence of forskolin, respectively). Suppression of apoptosis in RP-11 by forskolin was further documented immunocytochemically using TUNEL. As shown in Fig. 2B, *bottom*, forskolin substantially decreased the number of positively stained nuclei that contain fragmented DNA.

Fas-L is known to induce apoptosis in various cell types. In RP-11 cells, Fas-L (200 μg/ml) also induced apoptosis, which was completely reversed by forskolin treatment (Fig. 2C). Thus, in addition to its effect on serum deprivation-induced apoptosis, forskolin also suppressed apoptosis induced by physiological triggers, such as Fas-L.

Forskolin activates ERK, which is essential for cell survival in RP-11 periosteal cells. To study possible mechanisms involved in cAMP suppression of apoptosis, we first investigated the role of mitogen-activated protein kinases. Forskolin (10 μM) transiently increased ERK activity 12-fold, as measured by phosphorylation of ELK1, which peaked at 30 min (Fig. 3, *top*). Forskolin had little effect on JNK in these cells (Fig. 3, *center*). In contrast, forskolin decreased ERK activity in RCT-3 and C5.18 cells (Fig. 3, *bottom*), consistent with the lack of antiapoptotic effects in these cells.

PD98059, a selective inhibitor of MEK, binds specifically to this kinase and prevents its activation by upstream activators such as c-Raf (Alessi *et al.*, 1995) and has been used widely to evaluate the role of ERK in signaling pathways. Flow cytometric analysis showed that in RP-11 cells, PD98059 (0.5–5 μM) stimulated apoptosis dose-dependently without abolishing an antiapoptotic effect by forskolin (Fig. 4). These data suggest that ERK activity is essential for cell

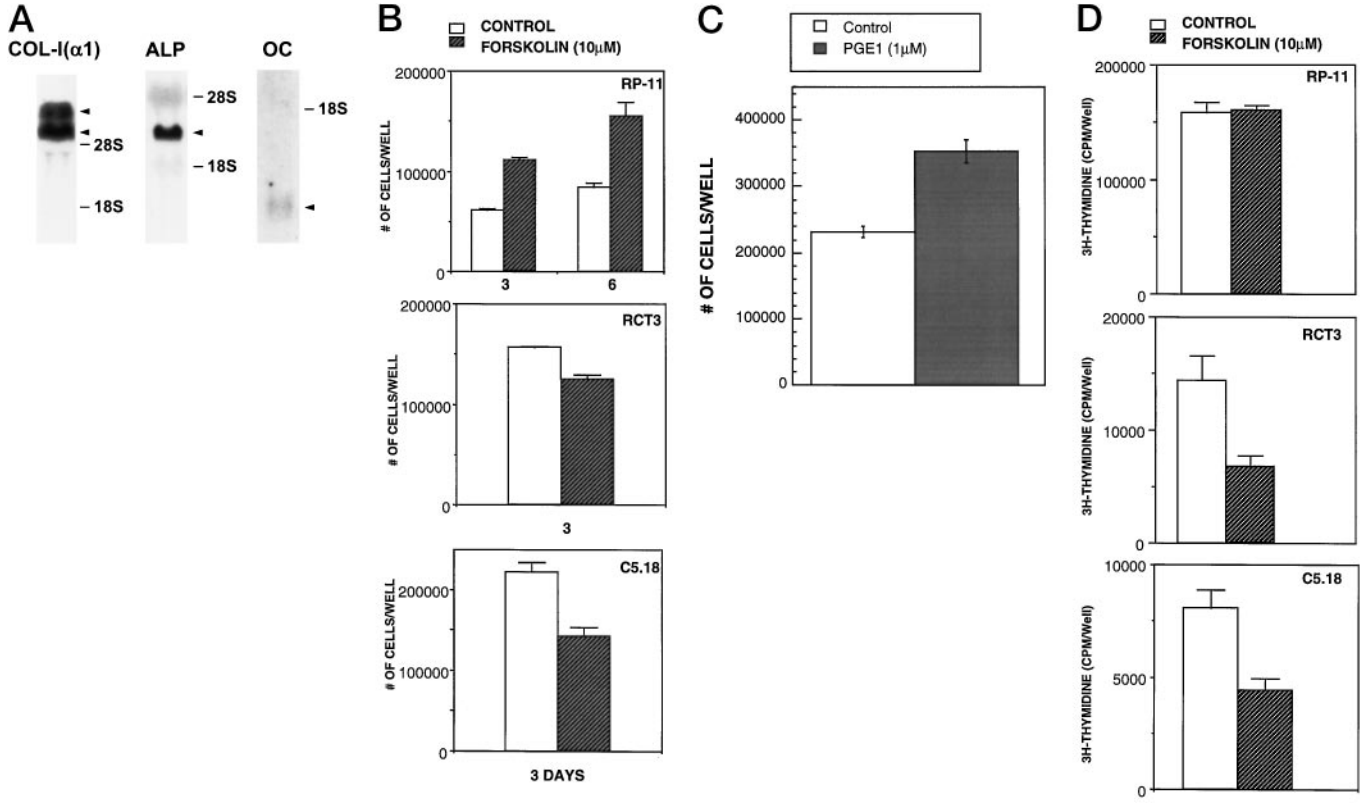

Fig. 1. Forskolin increases cell number but has no effect on proliferation in RP-11 periosteal cells. A, Nothern blot analysis of the expression of mRNA for ALP, type I collagen (α 1) and OC mRNAs in RP-11 periosteal cells. RP-11 cells express these three osteoblast phenotypic markers at confluence. B, Effect of forskolin on cell number in RP-11, RCT-3, and C5.18 cells. RP-11, RCT-3, and C5.18 cells were treated with forskolin (10 μM) in presence of 2% serum. Forskolin increased cell number in RP-11 cells by about 2-fold at 3 and 6 days, and decreased it in C5.18 and RCT-3. C, Effect of PGE1 on cell number in RP-11 cells. RP-11 cells were treated with PGE1 (1 μM) for 5 days. PGE1 increased RP-11 cell number. D, Effect of forskolin on [^{3}H]thymidine incorporation in RP-11, RCT-3, and C5.18 cells. RP-11, RCT-3, and C5.18 cells were serum-fasted for 24 hr and were treated with forskolin (10 μM) in the presence of 2% serum for 20 hr. [^{3}H]thymidine was added 2 hr before culture arrest. Forskolin had no effect on [^{3}H]thymidine incorporation in RP-11 cells, but decreased it in C5.18 and RCT-3. Data are mean ± standard deviation of quadruplicate determinations.

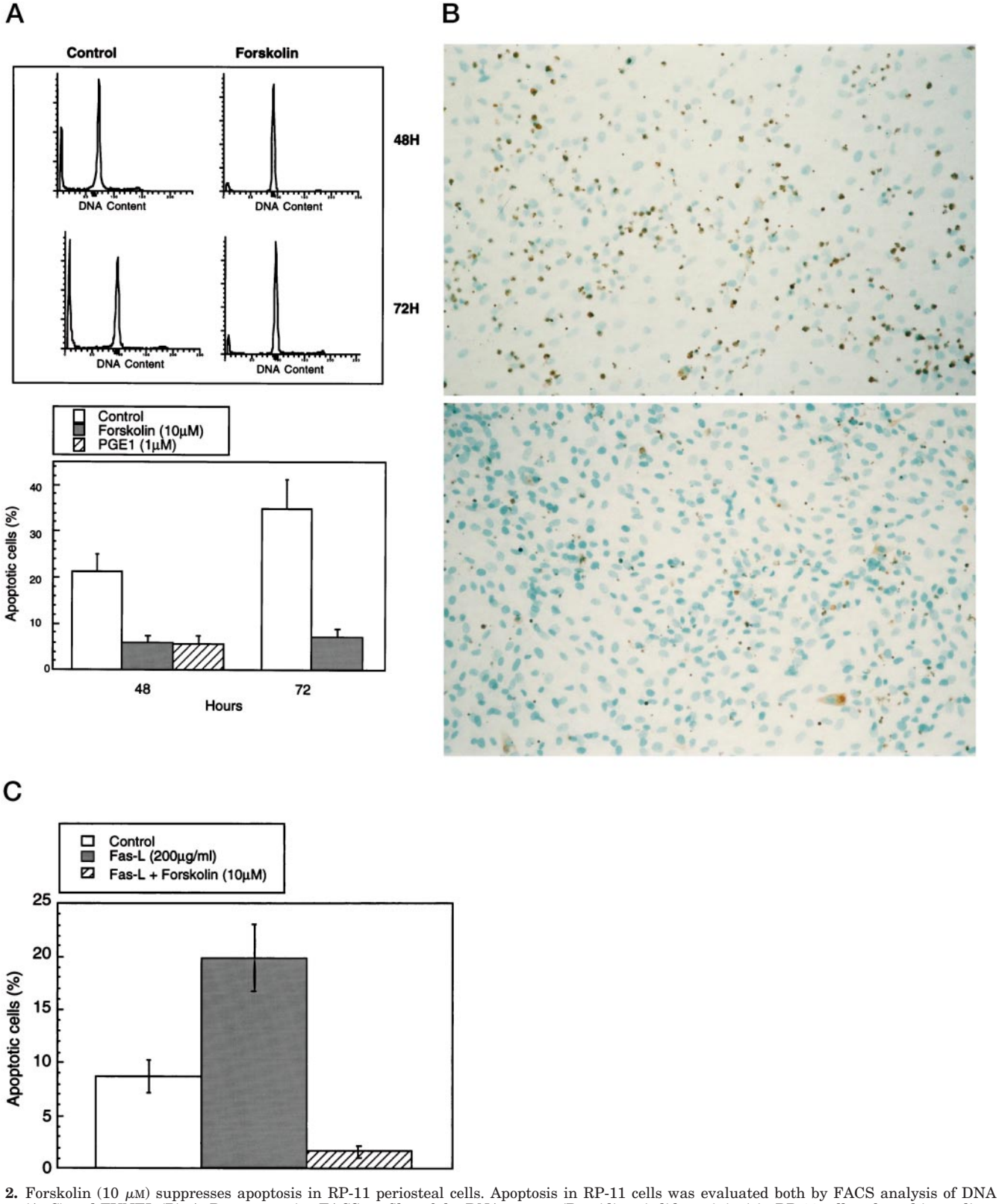

Fig. 2. Forskolin (10 μM) suppresses apoptosis in RP-11 periosteal cells. Apoptosis in RP-11 cells was evaluated both by FACS analysis of DNA content (A, C) and TUNEL (B). A, Representative FACS profiles of the DNA content (Propidium iodide staining) in RP-11 cells cultured in medium containing 2% serum in the presence or absence of forskolin (10 μM) for 48 and 72 hr. RP-11 cells display sub-G1 DNA content (*arrow head*, position of G1 peak) indicative of apoptosis. Quantification of the number of apoptotic cells, determined using ModFit software, shows that about 21% and 35% of cells undergo apoptosis by 48 and 72 hr, respectively. Treatment with forskolin suppressed apoptosis by about 72% and 79% at 48 and 72 hr, respectively. B, TUNEL staining of RP-11 cells cultured in medium containing 2% FBS in the absence (*top*) or presence (*bottom*) of forskolin for 48 hr. Cells were fixed in paraformaldehyde, permeabilized and incubated with the terminal deoxynucleotidyl transferase and dioxygenin-labeled UTP, to label free DNA 3′ ends. Labeled nuclei were detected using a horseradish peroxidase-coupled antidigoxygenin antibody. Magnification, 200 ×. C, RP-11 cells were treated for 24 hr with Fas-L (200 μg/ml) in the presence or absence of forskolin in culture medium containing 2% serum. The percentage of apoptotic cells was determined by flow cytometry as in A. Forskolin completely suppressed apoptosis induced by Fas-L.

survival, but forskolin suppression of apoptosis in RP-11 cells may involve another pathway.

Forskolin increases SPK activity in RP-11 cells in a cell type specific manner. *In vitro* kinase assays, using [γ-^{32}P]ATP and sphingosine as substrates, show that forskolin increases the cytosolic SPK activity in RP-11 cells up to 225%, peaking at 2 hr (Fig. 5, *top left*). dbcAMP (0.1–10 μM), a cAMP analog, also up-regulated SPK activity in these cells (Fig. 5, *top right*). In contrast, forskolin had no effect on SPK activity in RCT-3 and in C5.18 cells (Fig. 5, *bottom left*). Induction of SPK by cAMP in RP-11 cells (2.25-fold for forskolin) is modest compared with its effect on ERK (12-fold; Fig. 3, *top*) but is similar to the previously reported effects of PDGF (1.5-fold) (Olivera and Spiegel, 1993) and IgE (1.8 fold) (Choi *et al.*, 1996) on this enzymatic activity. SPP, a product of SPK, has been shown to induce ERK activity; however, in RP-11 cells, forskolin induction of ERK precedes the induction of SPK, which suggests an independent pathway. Indeed, DMS, a competitive inhibitor of SPK (Yatomi *et al.*, 1996), did not block forskolin induction of ERK activity (Fig. 5, *bottom right*), which suggests that, in RP-11 cells, forskolin induces ERK and SPK activities independently in a cell-type-specific manner.

SPK activation is required for the antiapoptotic effect of forskolin. To evaluate the role of the SPK pathway in the antiapoptotic effect of cAMP, we first examined the effect of SPP in RP-11 cells. SPP suppressed apoptosis in these cells in a dose-dependent manner (0.1–10 μM) (Fig. 6A), as previously reported in Swiss 3T3 fibroblasts (Cuvillier *et al.*, 1996). The fact that SPP had no effect on apoptosis in RP-11 cells at 0.01 μM suggests that, in these cells, SPP is not acting through the recently reported SPP cell surface receptor (Postma *et al.*, 1996). DMS, the natural *N*-methylated metabolite of SP, has been reported to potently and specifically inhibit SPK activity and SPP production both in cell culture and in *in vitro* kinase assays (Olivera and Spiegel, 1993; Hakomori and Igarashi, 1995). As shown in Fig. 6B, DMS completely suppressed the antiapoptotic effect of forskolin in a dose-dependent manner (0.01–1 μM). Co-treatment with the SPK product, SPP (10 μM), reversed the effect of DMS (Fig. 6C), which indicates that the effect of DMS on apoptosis was caused by inhibition of SPP production, in accordance with a specific inhibitory effect of DMS on SPK (Yatomi *et al.*, 1996). Unlike PD98059, DMS had no effect on apoptosis in the absence of forskolin, which suggests that, in RP-11 cells, SPK may play a specific role in cAMP-mediated suppression of apoptosis, whereas ERK is necessary for cell survival.

Finally, to examine the possible role of ERK in the antiapoptotic effect of SPK, cells were treated with SPP in the presence of PD98059. As shown in Fig. 6D, SPP still suppresses RP-11 cell apoptosis in the presence of PD98059, which suggests that the effect of SPP does not require an active ERK pathway. These results suggest that SPP suppression of apoptosis is mediated, at least in part, through a pathway separate from ERK.

Discussion

This study was prompted by the documented increase in periosteal bone formation produced by PGE1 and -E2 in humans (Ringel *et al.*, 1982) and animals (Jee *et al.*, 1987), which results mainly from an increase in the number of osteoblasts on the bone surface. The major PG receptor in bone is EP4, signaling via cAMP. It is therefore of interest that in rat periosteal cells in culture (cell line RP-11), cAMP increases cell number. The increase in cell number was not caused by cell proliferation but by suppression of apoptosis.

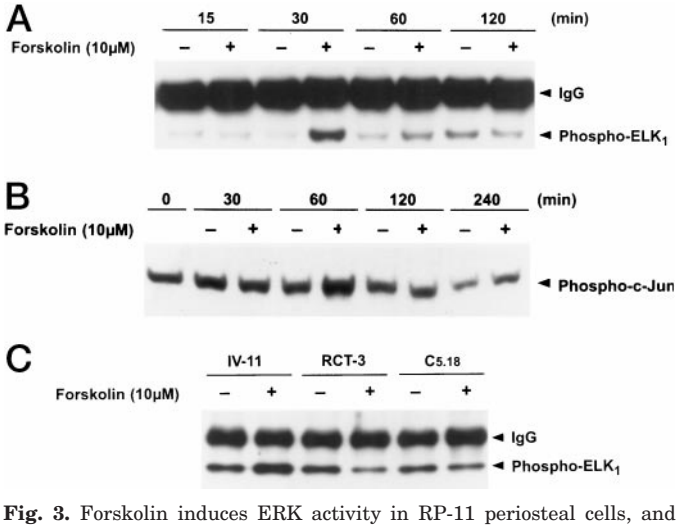

Fig. 3. Forskolin induces ERK activity in RP-11 periosteal cells, and suppresses it in RCT-3 and C5.18 calvaria-derived osteoblastic cells. ERK (*top*, *bottom*) and JNK (*center*) activities were determined by *in vitro* kinase assay after immunoprecipitation of phospho-ERK and "pull down" of JNK with a c-jun fusion protein, respectively. (*Top, center*) RP-11 cells were treated with vehicle or forskolin (10 μM) in the presence of 2% FBS for 15, 30, 60, 120, and 240 min. Forskolin dramatically induced ERK activity peaking at 30 min and decreasing thereafter. Forskolin modestly induced JNK activity peaking at 60 min after treatment. *Bottom*, RP-11, RCT-3, and C5.18 were treated with forskolin (10 μM) for 30 min. Forskolin increased ERK activity in RP-11 and decreased it in RCT-3 and C5.18 cells.

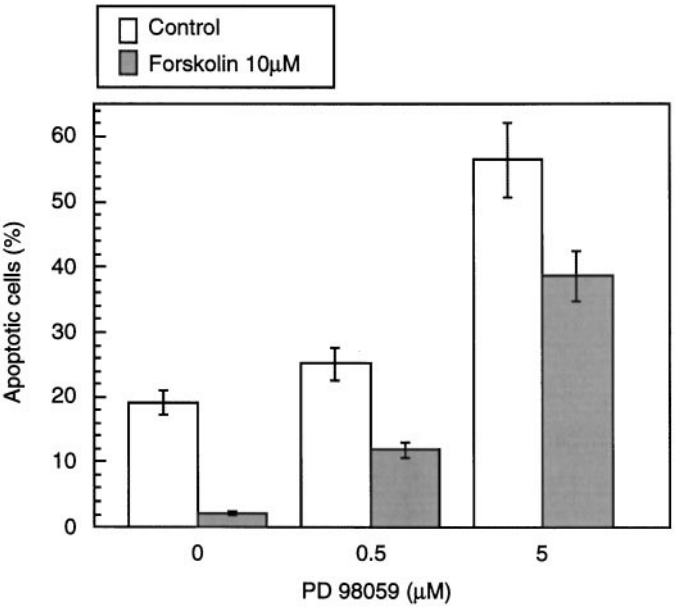

Fig. 4. PD98059, a MEK inhibitor, induces apoptosis in RP-11 cells. The number of apoptotic cells was determined using FACS analysis of DNA content, as described in Methods. RP-11 cells were cultured in medium containing 0, 0.5, and 5 μM PD98059 in the presence or absence of forskolin (10 μM) for 48 hr. PD98059 suppressed the antiapoptotic effect of forskolin in a dose-dependent manner. However, PD98059 also induced apoptosis in the absence of forskolin. Data are mean ± standard deviation of triplicate determinations.

The antiapoptotic effects of forskolin are cell-type specific, and were not observed in osteoblastic RCT-3 or chondro-osteogenic C5.18 cells, derived from rat calvaria. In these cells, forskolin suppressed proliferation consistent with previous reports showing that stimulation of intracellular cAMP suppresses proliferation in various rat and mouse calvaria-derived osteoblastic cells (Harada *et al.*, 1995). Interestingly, local treatment of adult rats with PGE2 induces bone formation in tibia but not in calvaria (Harada S, Rodan SB, Rodan GA, and Balena R, unpublished observations). These observations suggest that PGE2 and cAMP may have different effects on cells of calvaria and long bones, that may involve distinct intracellular pathways. In this study, we examined putative mechanisms for cAMP suppression of apoptosis in the RP-11 cell line. In neuronal PC12 cells, cAMP suppresses apoptosis through activation of ERK via B-Raf and the small G protein Rap1 (Vossler *et al.*, 1997). However, in rat fibroblasts, cAMP suppresses ERK activity (Wu *et al.*, 1993), which indicates the cell-type-specific character of this effect. We found that ERK activity is indeed up-regulated by cAMP in RP-11 cells but not in RCT-3 or C5.18 cells. Moreover, the mitogen-activated protein kinase kinase inhibitor PD98059 opposes the antiapoptotic effect of cAMP, which implies ERK participation in the cAMP effect. However, two observations suggest that ERK is not the major mediator of the antiapoptotic effect of cAMP in these cells: (i) PD98059 at concentrations of 0.5 and 5 μ M increased apoptosis independently of the presence of forskolin and (ii) in the presence of either concentration of PD98059, forskolin suppressed apoptosis by approximately the same extent (Fig. 4). Thus, although the

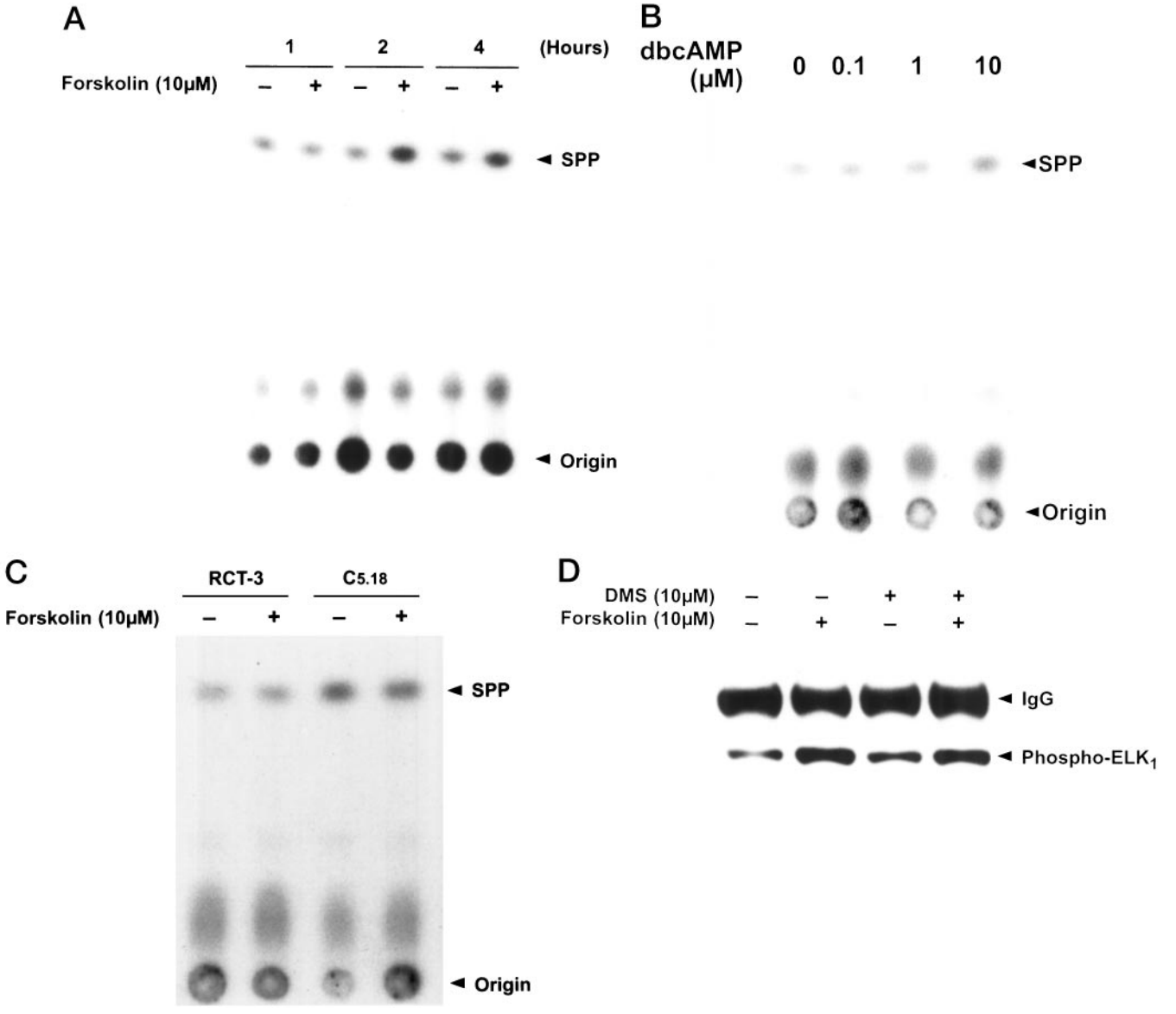

Fig. 5. SPK activity is induced by forskolin in RP-11 but not in RCT-3 and C5.18 cells. *Top*, RP-11 cells were treated with vehicle, forskolin (10 μM for 1, 2, and 4 hr), or dbcAMP (0.1, 1 and 10 μM for 2 hr) in the presence of 2% FBS. SPK activity was determined in the cytosolic cell fractions prepared as described in Methods using SP as substrate. Lipids were extracted and analyzed by thin layer chromatography. *Right arrowhead*, standard SPP, run simultaneously. Forskolin and dbcAMP increased SPK activity in RP-11 cells. *Bottom left,* RCT-3 and C5.18 cells were treated with vehicle or forskolin (10 μM) in the presence of 2% FBS for 2 hr. Forskolin had no effect on SPK activity in RCT-3 and C5.18 cells. *Bottom right*, ERK activity was determined after immunoprecipitation with phospho-ERK antibody and *in vitro* kinase assay using ELK_1 as substrate, as described in Methods. RP-11 cells were treated with vehicle or forskolin (10 μM) in the presence or absence of 10 μM DMS for 30 min.

specific up-regulation of ERK activity by cAMP is interesting and should be investigated further, these findings suggest another mediator for the antiapoptotic effects of cAMP.

Ceramide and its metabolites have recently been implicated in the regulation of apoptosis. Apoptotic stimuli, such as Fas-L, TNFα, γ-interferon, and hypoxia, increase intracellular ceramide levels (reviewed in Hannun and Obeid, 1995). Furthermore, membrane permeable ceramide analogs induce apoptosis in U937 and HL-60 cell lines (Cuvillier *et al.*, 1996). On the other hand, SPP, a ceramide metabolite, prevents apoptosis (Cuvillier *et al.*, 1996), which suggests that the balance between ceramide and SPP may control this process. The metabolic pathway that controls the intracellular levels of the two products includes ceramidase, which cleaves ceramide to SP, and SPK, which phosphorylates SP to SPP. Regulation of these enzymes has not been studied extensively. In fibroblasts, using SP as substrate, SPK was shown to be activated by PDGF, serum (Olivera and Spiegel, 1993) and 12-*O*-tetradecanoylphorbol-13-acetate (Cuvillier *et al.*, 1996). SPK activity was not up-regulated by other mitogens, including epidermal growth factor, insulin, bombesin, and bradykinin, indicating selectivity for its stimulation. SPK activity is also increased in mast cells by IgE via the high affinity IgE receptor, Fcϵ RI (Choi *et al.*, 1996).

As far as we know, the present study is the first report on stimulation of SPK activity by cAMP. cAMP stimulation is

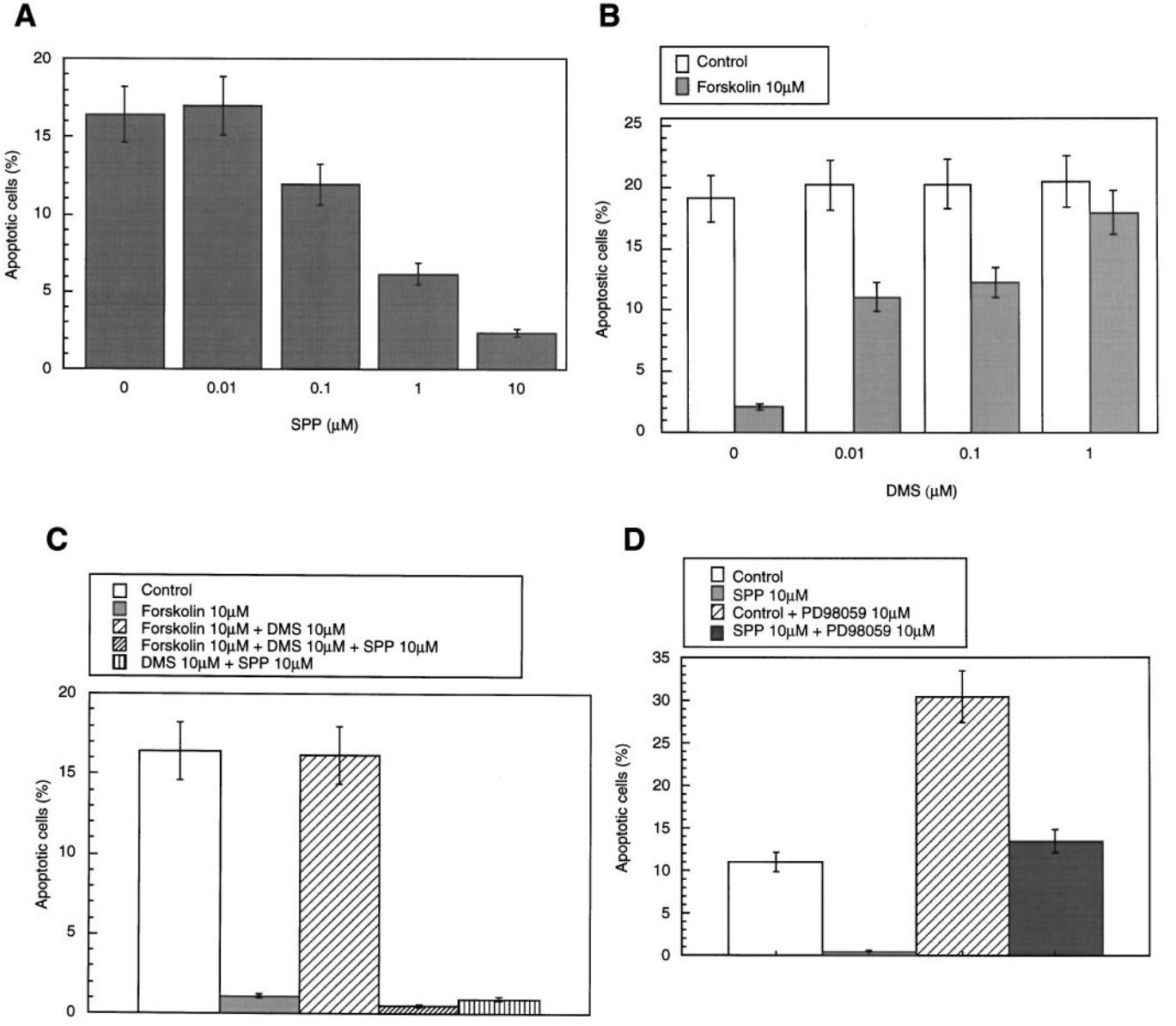

Fig. 6. SPP suppresses apoptosis, and DMS reverses the forskolin antiapoptotic effect in a dose-dependent manner. The number of apoptotic cells was determined using FACS analysis of DNA content, as described in Methods. A, RP-11 periosteal cells were cultured in medium containing 2% FBS and increasing concentrations of SPP (0, 0.01, 0.1, 1, and 10 μM) for 48 hr. B, RP-11 periosteal cells were cultured in medium containing 0, 0.01, 0.1, and 1 μM DMS in the presence or absence of forskolin (10 μM) for 48 hr. DMS suppressed the antiapoptotic effect of forskolin in a dose-dependent manner; however, DMS did not induce apoptosis in the absence of forskolin. C, RP-11 periosteal cells were treated with combinations of 10 μM forskolin, 10 μM DMS, and 10 μM SPP, for 48 hr, as indicated. DMS induction of apoptosis in the presence of forskolin was reversed by co-treatment with SPP. D, RP-11 periosteal cells were treated with 10 μM SPP and/or 10 μM PD98059 for 48 hr. SPP suppressed apoptosis in the presence of PD98059. Data are mean $\pm$ standard deviation of triplicate determinations.

cell-type specific, observed in rat periosteal cells (RP-11) but not in calvaria-derived bone cells, and correlates with cAMP effects on apoptosis in these cells. The pharmacological data presented in this study strongly suggest that the SPK product, SPP, mediates the antiapoptotic affects of cAMP. The methyl derivative of SP, DMS, a potent inhibitor of SPK (Yatomi *et al.*, 1996), abolished the antiapoptotic effects of cAMP, and its action was reversed by the exogenous addition of SPP, the SPK product. Unlike the MEK inhibitor PD98059, DMS, up to 1 μM, did not induce apoptosis on its own, in the absence of cAMP. Taken together, these results support a specific role for SPK and SPP in cAMP-mediated suppression of apoptosis in RP-11 cells. SPK has not yet been purified or cloned, so its molecular identification should allow further characterization of this pathway. Interestingly, SPP was reported to induce ERK activity in U937 monoblastic leukemia cells (Cuvillier *et al.*, 1996). However, in this study, forskolin effects on ERK precede its effects on SPK, and the SPK inhibitor has no detectable effect on ERK activation by cAMP (Fig. 5, *bottom right*) in our assay, which suggests that the SPP action is mediated, at least in part, by a pathway independent of ERK. The downstream targets of SPP are not known at this point. In U937 cells, SPP was reported to activate NFκB (Shatrov *et al.*, 1997); NFκB plays a role in the survival of macrophages (Beg and Baltimore, 1996). Further studies are needed to evaluate whether NFκB is a downstream mediator of SPP in RP-11 cells.

In summary, we report in this study a rare cAMP suppressive effect on apoptosis, which seems to be mediated by SPP, generated by cell-specific cAMP up-regulation of SPK activity.

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Send reprint requests to: Dr. Shun-ichi Harada, Department of Bone Biology and Osteoporosis Research, Merck Research Laboratories, WP26A-1000, West Point, PA 19486. E-mail: harada@merck.com