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Multiple Intracellular Pathways Interfere with the Activation of a CPP32-like Protease Induced by Serum Deprivation of AKR-2B Cells

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As previously described, confluent AKR-2B fibroblasts rapidly disintegrate upon removal of serum. Platelet-derived growth factor isoforms AB or BB (PDGF-AB, -BB) added immediately after serum deprivation caused complete survival of the cells without initiating proliferation (Simm *et al.*, 1994, *J. Cell. Physiol.* 160, 295). Here the role of cAMP as a protective agent was investigated by using forskolin or 8-Br-cAMP. Both reagents afforded high cellular protection. The phorbolester TPA, an activator of protein kinase C isoforms, also exerted a high protection against cell death (ED_{50} = 7 nM). Unexpectedly colchicine (ED_{50} = 1.5 μM) an inhibitor of tubulin polymerization also protected cells from death. The protective effects of PDGF-BB and TPA were dependent on protein synthesis as indicated by their complete suppression by cycloheximide (CHx). Surprisingly, forskolin and 8-Br-cAMP remained effective even in the presence of CHx. Detailed studies of several signalling pathways were performed. These investigations showed no interference between PDGF-BB and cAMP-dependent pathways at the early stage of signal transduction. As previously described, the ICE-like protease inhibitor tyr-val-ala-asp-chloromethylketone (YVAD-cmk) protected cells from death (Simm *et al.*, 1997, *J. Cell Sci.* 110, 819–828). As shown here, a substantial protection was also achieved by the addition of two other caspase inhibitors: asp-glu-val-asp-aldehyde (DEVD-cho; ED_{50} = 100 μM) and benzoylcarbonyl-asp-glu-val-asp-chloromethylketone (Z-DEVD-cmk; ED_{50} = 100 μM). The activity of caspases was studied using either tyr-val-ala-asp-aminomethylcoumarine (YVAD-amc) or asp-glu-val-asp-aminomethylcoumarine (DEVD-amc) as substrates. There was no activation of a YVADase, whereas as pronounced increase in DEVDase activity was found with a maximum 3 h after serum removal. Cross competition experiments *in vitro* showed that the latter activity is inhibited also by low concentrations of YVAD-cmk (300–600 nM), suggesting that both inhibitors inactivated the same target protease. Remarkably all tested protective reagents lead to an inhibition of the DEVDase activity in intact cells. Since these reagents act via distinct intracellular pathways, the existence of a regulatory element upstream of the DEVDase is proposed which integrates signals from a variety of pathways. © 1998 Academic Press

INTRODUCTION

Selective cell death plays an important role in determining population size in specific groups of cells. The survival of many types of cells is critically dependent on the presence of specific growth factors or yet undefined serum components. In contrast to necrosis, which is a consequence of the plasma membrane disruption due to chemical or physical insult, that kind of cell death, termed apoptosis, is an active process of the target cell. Apoptosis appears in many facets, but certain morphological and biochemical changes are recurrent. For most of the investigated cells the early events of cell death are characterized by a condensation of chromatin followed by a fragmentation of the DNA yielding a "DNA ladder" (for review see [1–11]). The collapsed nucleus frequently breaks up into spheres forming "apoptotic bodies." Finally these particles are engulfed either by macrophages or by neighbouring cells.

The emerging view of apoptosis is that diverse regulatory pathways activate a common execution machinery which carries out cell disassembly. Although this execution machinery is poorly understood, it appears that an essential component is the caspases family, cysteine proteases which are activated by proteolytic cleavage from precursor proteins. Caspases cleave their substrates after an aspartate residue, a very unusual substrate specificity for eukaryotic proteases. This family of enzymes consisting of caspase-1 (ICE),[2]

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[2] Abbreviations used: PDGF, platelet-derived growth factor; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; EGTA, ethylene-glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; kemptide, a peptide with the sequence leu-arg-arg-ala-ser-leu-gly; PKI has the sequence gly-arg-thr-arg-arg-asn-

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caspase-2 (ICH-1), caspase-3 (CPP32/Yama/apopain), caspase-4 (ICE-relII/TX/ICH-2), caspase-5 (ICErel-III/TY), caspase-6 (Mch2), caspase-7 (Mch3/ICE-LAP3/CMH-1), caspase-8 (FLICE/MACH/Mch5), caspase-9 (ICE-LAP/Mch6), caspase-10 (Mch4), caspase-11, and caspase-12 has been suggested to play a central part in the apoptotic process [12–14].

On the other hand, the disruption of the mitochondrial inner transmembrane potential ($\Delta\psi_m$) marks a point of no return for the apoptotic cascade. Two mitochondrial proapoptotic factors have been purified: the 15-kDa cytochrome c protein which acts together with cytosolic factors [15–17], and a 50-kDa protease that by itself suffices to cause nuclear apoptosis [18].

Another family of proteins whose function in regulating apoptosis is phylogenetically conserved is the human Bcl-2 protein family [19]. Some members of this family, e.g., Bcl-2, Bcl-X_L, Ced-9, and the adenovirus E1B 19-kDa protein are able to inhibit apoptosis. Other members, such as Bax, Bad, Bcl-X_S, and Bak have the opposing effect of promoting cell death. Most recently the interaction of some members of this family with signal transduction components related to proliferation control have been identified shedding some light on the mechanism by which growth factors and associated signaling pathways may prevent apoptosis [11, 20, 21].

Density-arrested fibroblasts like Balbc/3T3 or AKR-2B cells die after serum deprivation. Depending on the cell line, death after serum removal is either immediately initiated (Balbc/3T3) [22–25] or starts after a delay of 90 min (AKR-2B) [26, 27]. Dying of the cells ceases after 5–6 h with a survival of 10–20% (Balbc/3T3) or 50% (AKR-2B). Morphological changes are quite similar for both cell lines including membrane blebbing and chromatin condensation. These are typical characteristics for apoptosis, but remarkably there is no DNA fragmentation. Furthermore both cell types die by membrane disrupture. This unusual behavior has been recently characterized in detail for AKR-2B cells [27]. Detailed studies on second messenger-related pathways that prevent cell death have been presented by Tamm and coworkers using Balbc/3T3 cells [22–25]. These authors found that activation of both PKA-, and PKC-dependent pathways protect cells from death. They furthermore reached the conclusion that activation of the cAMP pathway rather than that of the protein kinase C is responsible for the protective effect elicited by PDGF [23, 25]. In agreement with the results from Tamm and coworkers using Balbc/3T3 cells we describe here that PKA- and PKC-dependent pathways play a role in the protection of AKR-2B fibroblasts. However, in AKR-2B cells PDGF-BB does not lead to an activation of the cAMP-dependent pathway. In a recent publication we have demonstrated that ICE-like proteases are possibly involved in the mechanism of cell death found with AKR-2B cells. In an attempt to identify the responsible caspase(s) we used two fluorogenic substrates (YVAD-amc and DEVD-amc). DEVD-amc corresponds to the apoptotic cleavage site of PARP, a putative natural substrate of CPP32, whereas YVAD-amc is derived from the cleavage site preferred by caspase-1 (ICE). There was no activation of a YVADase. However, a pronounced increase in DEVD-amc cleaving activity was found reaching a maximum after 3 h of serum deprivation. The parallelity in the time dependence of cell disintegration and activation of this protease suggests its involvement in the execution of cell death. We furthermore show that multiple second messenger related pathways interfere with the activation of this protease. The existence of a regulatory element upstream of this protease is suggested which integrates signals from a variety of intracellular pathways.

MATERIALS AND METHODS

PDGF was prepared as described [28, 29]. Cell culture reagents were purchased from Gibco (Eggenstein, Germany). [γ-^{32}P]ATP (3000 Ci/mmol) was from Hartmann Analytical (Braunschweig, Germany). TPA, forskolin, cycloheximide, and FITC-labeled phalloidin were from Sigma (Deisenhofen, Germany). 8-Br-cAMP was from Biolog (Bremen, Germany). PKI peptide inhibitor was from Natutec (Frankfurt, Germany). YVAD-cmk, YVAD-amc, DEVD-cho, Z-DEVD-cmk, and DEVD-amc were from Bachem (Heidelberg, Germany); AMC was purchased from Fluka (Buchs, Switzerland). The antibody against ICE-protease (ICE-p20 (M-19)) was from Santa Cruz (Heidelberg, Germany).

Cell number. A subclone DT1 of AKR-2B mouse fibroblasts was used. Various methods including microscopy and flow cytometry did not show any heterogeneity among the cells. Stock cultures were propagated in antibiotic-free McCoy-5A medium with 5% Hyclone calf serum for less than 3 months to minimize fluctuations. During that time responses to PDGF did not change. Cells were prepared for the stimulation with various reagents using the protocol originally described in [30, 31]. Briefly, experimental cultures were seeded at a density of 5×10^3 cells/cm^2 into 24-well plastic dishes (Falcon) and grown for 5 days in McCoy-5A medium containing 5% calf serum (Hyclone) without a medium change. For the measurement of cell survival, cells were washed twice with serum-free MCDB 402 medium (starvation medium) and kept in this medium for the indicated time period. Immediately after serum removal, the respective tested compounds were added. The number of viable cells was determined using the CASY-1 system (Schärfe, Reutlingen, Germany) based on the Coulter Counter principle.

Determination of MAP-kinase activities after ion-exchange chromatography. Preparation of cytosolic extracts and determination of MAP kinase activity was performed essentially as described [32–34]. Cells were grown in 10 150-mm culture dishes (Greiner). Stim-

ala-ile-his-asp-NH_2; PKA, cAMP-dependent protein kinase; PKB, protein kinase B; PKC, protein kinase C; ICE, interleukin-1β-converting enzyme; TPA, phorbol-12-myristate-13-acetate; YVAD-cmk, acetyl-tyr-val-ala-asp-chloromethylketone; DEVD-cho, acetyl-asp-glu-val-asp-aldehyde; Z-DEVD-cmk, benzoylcarbonyl-asp-glu-val-asp-chloromethylketone; YVAD-amc, acetyl-tyr-val-ala-asp-amino-methy-coumarine; DEVD-amc, acetyl-asp-glu-val-asp-aminomethyl-coumarine; CHx, cycloheximide; CS, calf serum; PI(3)-kinase, phosphatidylinositol(3)-kinase; PARP, poly(ADP-ribose)polymerase.

ulation was done for 10 min. Cells were then rinsed twice with 5 ml ice-cold phosphate-buffered saline and once with 5 ml of ice-cold extraction buffer containing 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 μg/ml leupeptine, and 1 mM phenylmethanesulfonylfluoride (PMSF). Cells were scraped into a total volume of about 0.7 ml extraction buffer and were disrupted with a micro ultra turrax and then centrifuged at 100,000*g* for 20 min at 4°C. Chromatographic separation was carried out at 4°C using a Merck inert HPLC-system with a Resource Q column for anion exchange chromatography from Pharmacia. Two milligrams of extract were fractioned on this column equilibrated with 50 mM β-glycerophosphate, pH 7.3, 1 mM EGTA, 0.1 mM sodium orthovanadate, and 1 mM dithiothreitol (buffer A). After sample application the column was washed at a flow rate of 0.5 ml/min for 10 min. Then a linear gradient from buffer A to buffer A-containing 300 mM NaCl was applied during 45 min at a flow rate of 0.5 ml/min. Ninety six fractions were collected during the entire run.

Assays were performed in 96-well microtiter plates (V-form). The column fraction (12.5 μl) was mixed with 4.2 μl of myelin basic protein (2 mg/ml), and the reaction was started by the addition of 10 μl assay buffer containing 75 mM β-glycerophosphate, pH 7.3, 0.15 mM sodium orthovanadate, 1.5 mM dithiothreitol, 30 μM calmidazolium, 30 mM $MgCl_2$, and 0.3 mM [γ-^{32}P]ATP (specific activity $\sim$ 500 cpm/pmol). The reaction was stopped by the addition of each 10 μl of 0.5 M phosphoric acid. Twenty microliters was spotted onto P81 phosphocellulose. After washing four times with 175 mM phosphoric acid, radioactivity was determined by using a phosphoimager.

Determination of cAMP concentration and cAMP-dependent protein kinase activity. For determination of the cAMP contents, cells were grown in 6-well plates. For termination of the reaction, cells were washed briefly with MCDB medium and subsequently lysed in 60% methanol. After filtration through reversed phase chromatography material Nucleosil C18 7 μm (Macherey and Nagel, Düren, Germany) the filtrate was dried and the residue was taken up in 40 μl water. The cAMP content in this extract was determined with the kit from Amersham (Braunschweig, Germany). Cell number and volume were determined with the CASY-1 system, thus allowing the calculation of the intracellular cAMP content.

PKA activity was assayed by measuring the phosphorylation of kemptide (0.17 mM) in the absence or presence of PKI peptide (5 μM). PKA activity was calculated as the amount of kemptide phosphorylated in the absence of PKI peptide minus that phosphorylated in the presence of PKI peptide. Cell extracts were prepared as described.

Determination of caspase activity. All steps were performed at 4°C. Cells grown on 10-cm culture dishes were rinsed in 10 ml PBS and scraped in 200 μl extraction buffer (50 mM Hepes–NaOH, pH 7.0, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF). They were disrupted with a micro ultra turrax and homogenate was clarified by a 20-min centrifugation at 100,000*g*. The supernatant was frozen at −80°C. All experiments described in the present study were performed within 1 week after preparation of the extract.

Fifty micrograms of extract (determined by the BCA method) were diluted in ICE standard buffer (100 mM Hepes–KOH, pH 7.5, 10% sucrose, 0.1% Chaps, 10 mM DTT, 0.1 mg/ml BSA). The enzymatic reactions were carried out in a total volume of 2 ml containing 15 μM YVAD-amc or DEVD-amc. Release of amc was monitored over a time period of 20 min in a fluorospectrophotometer [Fluoromax, SPEX] at room temperature with excitation and emission wavelength of 380 and 440 nm, respectively. During that time the fluorescence increase was linear, as shown in Fig. 6B. From the slope, the activity was calculated and the amount of fluorochrome was determined by comparison to an amc standard curve prepared in ICE standard buffer. For the determination of background activities, 250 nM of the inhibitor for ICE (YVAD-cmk) or CPP32 (DEVD-cho) was added to the reaction mixture.

Loading the cells with DEVD-cho or Z-DEVD-cmk. AKR-2B cells were grown as described in 24-well plates. Cells were preincubated at 37°C in hypotonic medium (1:8 dilution of MCDB 402) supplemented with increasing concentrations of either DEVD-cho or Z-DEVD-cmk. After 2 min the hypotonic medium was replaced by MCDB 402 supplemented with respective concentrations of inhibitor. Alternatively, cells were preincubated with DEVD-cho or Z-DEVD-cmk, respectively, in McCoy + 5% CS for 5 h before medium was replaced by MCDB 402 containing the indicated amount of the respective inhibitor.

Staining of the cytoskeleton. Cells were grown to confluency on glass cover slips in McCoy-5A medium containing 5% calf serum (Hyclone). After treatment with the indicated reagents for the respective time intervals cells were washed twice with PBS and were fixed for 20 min in 3% paraformaldehyd in PBS. This solution was removed and the cells were incubated in 0.1%. Triton X-100 in PBS for 5 min. After washing twice with PBS, the cells were incubated with one drop of FITC-labeled phalloidin (50 μg/ml in water) for 30 min. After washing twice with PBS, cells were mounted with the antifading medium from Molecular Probes. The microscope DMIRB from Leica (Bensheim, Germany) was used. Magnification was 630-fold and the filter set I3 was used.

Immunoblotting was performed as described [26].

Statistics. Cell numbers were determined in triplicates from independent cultures. The bars represent standard errors. All experiments were done three times. For immunoblotting, immunostaining, measurements of caspase activities or protein kinase activities, typical results from three independent experiments are shown.

RESULTS

Activation of Multiple Pathways Affords Protection

As previously shown, AKR-2B cells grown in McCoy medium in the presence of 5% Hyclone calf serum reach confluency and final density after 4–5 days. In the standard protocol originally designed by Shipley, this medium is then exchanged to serum- and protein-free MCDB 402 medium (31). After this change, approximately 50% of the cells die following a rapid exponential decay with a half-life time of 3.5 h. A recently performed more detailed study showed that cell death did not occur immediately after medium change but with a delay of about 90 min (27). It has been recognized for some time that hormones or growth factors coupled to intracellular pathways might be involved in the protection of cells after serum deprivation. Figure 1 shows that forskolin, an activator of the adenylate cyclase 8-Br-cAMP, and TPA, an activator of PKC, as well as PDGF-BB, are potent reagents exerting significant protection against cell death. Unexpectedly, colchicine, which so far has been reported to promote apoptosis [35, 36], afforded strong protection against cell disintegration.

As shown previously [26, 27] the process of cell dying is almost complete after 6 h since cell number does not change significantly later on. However, there are some differences found with various agents regarding their effectiveness to protect for short term (6 h) or long term (24 h) treatments. It is clear from Fig. 1 that PDGF-BB protects the cells completely over the entire time

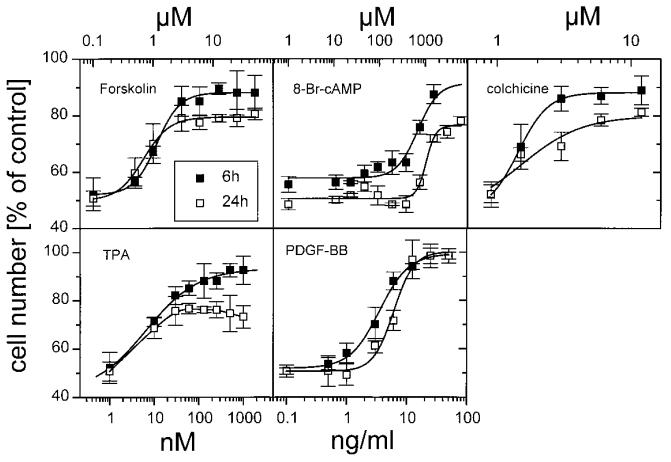

FIG. 1. Concentration dependency of cell survival induced by various agents. AKR-2B cells were grown in McCoy medium supplemented with 5% Hyclone calf serum to a final density of 250,000 cells/cm^2. Cells were shifted to MCDB 402 medium and immediately after, the indicated amounts of reagents were added. Cell number was determined after 6 and 24 h. For control (100%) cells were left in McCoy medium containing 5% calf serum. About 50% of the cells died when MCDB 402 medium was added without further supplements. Curves were fitted according to a logistic four parameters model contained in the program "origin" (Microcal). ED_{50} values (6 h incubation time) were 1.15 ± 0.09 μM for forskolin, 6.7 ± 3.5 nM for TPA, 685 ± 85 μM for 8-Br-cAMP, 3.5 ± 0.33 ng/ml for PDGF-BB, and 1.5 ± 0.3 μM for colchicine.

interval. However, the other protective reagents are less effective after 24 h (~80% remaining cells). Similar ED_{50} values were observed for both time intervals for each respective reagent.

Different Requirement of Protein Synthesis

As previously discussed [22, 23, 25], protein synthesis might be involved to different extents in the protection by various reagents. We therefore tested the protective reagents PDGF-BB, TPA, forskolin, and 8-Br-cAMP for their potency in the presence or absence of the protein synthesis inhibitor cycloheximide (CHx). In Fig. 2 the results after 6 and 24 h are compared. CHx alone had some effect after 6 h and drastically reduced the number of living cells after 24 h below control values. The ED_{50}-value for CHx was 5 μg/ml. The effect was maximum at concentration above 50 μg/ml (data not shown). There was a detectable decrease in cell number after 6 h when PDGF or TPA, respectively, were added together with CHx. On the contrary, the effect of CHx on cells treated with either forskolin or 8-Br-cAMP was very small after 6 h. More drastic effects were seen after 24 h. In the case of PDGF or TPA the treatment with CHx lead to cell numbers below control. But CHx had no influence on the protective effect of forskolin. The protective effect of 8-Br-cAMP was only partially reversed by CHx-treatment. These data gave thus the first hint that the protective effect of PDGF-BB does not involve a cAMP-dependent pathway.

PDGF and cAMP Act via Distinct Pathways

Tamm and coworkers have previously shown that in Balbc/3T3 cells the protective effect of PDGF is partly due to an activation of the cAMP-dependent pathway [22, 23, 25]. In a recent publication this pathway was delineated in more detail for smooth muscle cells [37]. Since there are considerable differences in the appearance and mechanism of cell death in various cell culture systems, we reanalyzed whether there is an interference of PDGF and forskolin-induced pathways in AKR-2B cells. Figure 3 shows the effects of both reagents on the accumulation of cAMP in the cell and on the activation of PKA. There was a massive increase in these two investigated parameters shortly after the addition of 50 μM forskolin. No increase was observed after PDGF-BB treatment, even after 4 h (data not shown).

It has been demonstrated in various cell types that raising the intracellular concentration of cAMP leads to phosphorylation and concomitant inactivation of the raf-1 kinase. As a consequence, a strong inhibition of the ras-raf-MAP-kinase pathway occurred which could be monitored by measuring the MAP-kinase activity [38–40]. Instead, as shown in Fig. 4 there was no cross-

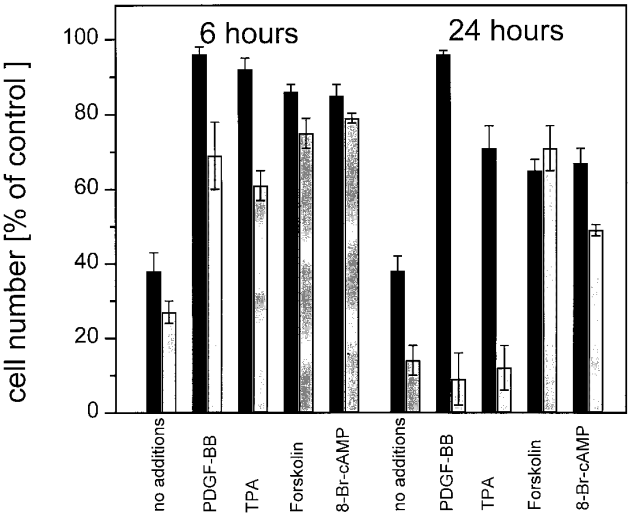

FIG. 2. Protective effect of various reagents in the presence or absence of the protein synthesis inhibitor cycloheximide (CHx). After removal of serum cells were incubated with indicated amounts of reagents. Concentrations that gave maximum protective effects were chosen (PDGF-BB, 50 ng/ml; TPA, 1 μM; forskolin, 10 μM; and 8-Br-cAMP, 1.5 mM). Black columns, without CHx; grey columns, in the presence of 50 μg/ml CHx. Cell number was determined after 6 and 24 h. Control cells were kept in McCoy medium containing 5% calf serum.

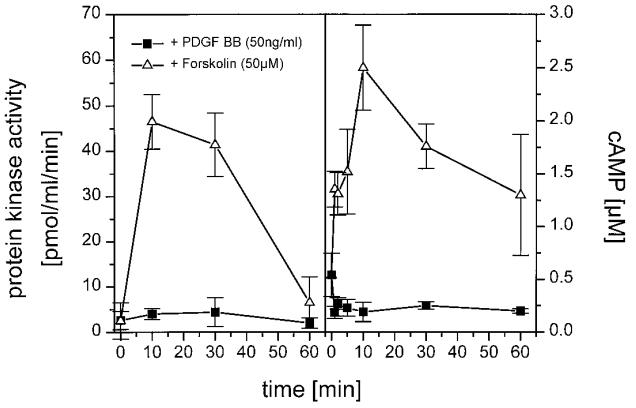

FIG. 3. cAMP content and cAMP-dependent protein kinase activity after treatment with PDGF-BB or forskolin. AKR-2B cells were grown as described. McCoy medium was replaced by MCDB 402 and the factors were added immediately after. After the indicated times, cells were lysed to determine the intracellular cAMP content (right panel) and the activity of cAMP-dependent protein kinases (left panel).

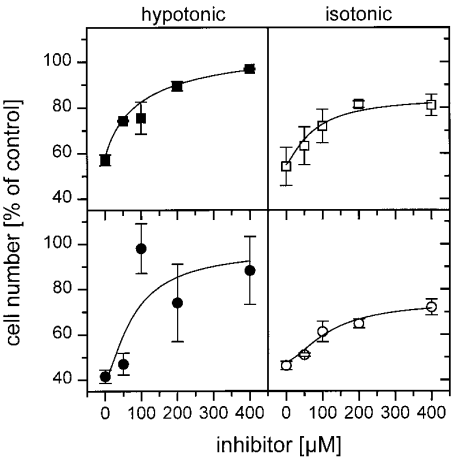

FIG. 5. Inhibition of cell death by DEVD-cho or Z-DEVD-cmk. AKR-2B cells were loaded with increasing concentrations of the inhibitor DEVD-cho (circles) or Z-DEVD-cmk (squares), respectively, by an hypotonic shock (closed symbols) or preincubation under isotonic conditions (open symbols). Cells were than incubated in MCDB 402 supplemented with the respective concentrations of inhibitor. After 6 h the number of viable cells was determined.

talk of cAMP with this pathway, since neither forskolin alone activated MAP-kinase (ERK-I and -II) nor was there a significant inhibition of the PDGF-BB induced stimulation. Thus, it seems unlikely that PDGF-BB- and cAMP-dependent pathways are coupled in AKR-2B cells.

Inhibition of Cell Death by DEVD-cho or Z-DEVD-cmk

Various experiments have shown that the amino acid sequence DEVD is the substrate for caspases belonging to the CPP32-like family (41). It is also well established that during apoptosis there is a cascade of caspases involved that leads to cell disintegration. It is postulated that processing of an ICE-like caspase is followed by the activation of a CPP32-like caspase, which in turn leads to cleavages of several substrates. As AKR-2B fibroblasts undergo apoptosis after serum removal, which can be prevented using the caspase 1 inhibitor YVAD-cmk (27), we studied the effect of the potent inhibitors of CPP32-like caspases DEVD-cho or Z-DEVD-cmk on cell survival of AKR-2B fibroblasts after serum removal. Since DEVD-cho is supposed to not be able to penetrate the cell membrane efficiently, two different approaches for loading the cells with either inhibitors were applied. In one experiment the fibroblasts were permeabilized in the presence of the indicated inhibitor concentrations using an hypoosmotic shock procedure in serum-free medium. Cells were counted 6 h later. As shown in Fig. 5, maximum protection from cell death was reached at 400 μM of both inhibitors with 90% of surviving cells. The ED_{50} was about 100 μM for both inhibitors. In the second approach, AKR-2B fibroblasts were preincubated with either DEVD-cho or Z-DEVD-cmk, 5 h before serum removal without hypotonic shock application and further treated for 6 h with the indicated concentrations of DEVD-cho or Z-DEVD-cmk in serum-free medium. As shown in Fig. 5, again a significant cell protection was achieved with an ED_{50} of about 100 μM for both inhibitors. Z-DEVD-cmk was slightly more effective leading to 80% surviving cells compared to 70% found with DEVD-cho.

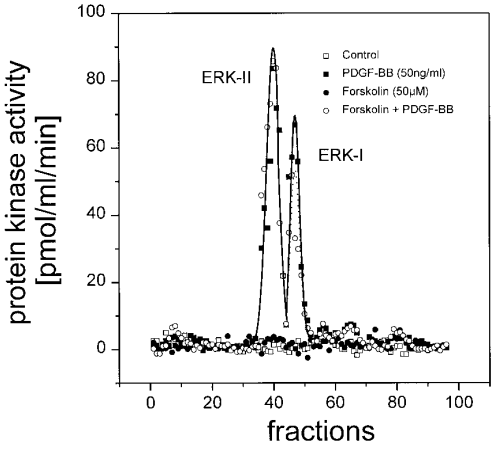

FIG. 4. MAP kinase activity in AKR-2B cells after treatment with PDGF-BB or forskolin. Cells were grown in 14-cm-diameter dishes to a final density of 250,000 cells/cm^2 in McCoy medium containing 5% calf serum. The medium was replaced by MCDB 402 containing the indicated amounts of reagents. Cells were lysed after 10 min and a cytosolic fraction was prepared. 2 mg of protein were chromatographed on a Resource Q column as described under Materials and Methods. 96 fractions were collected and the MAP kinase activity was determined using myelin basic protein as substrate.

A CPP 32-like Caspase Is Activated during Cell Death of AKR-2B Fibroblasts

In order to delineate the involvement of an ICE-like protease in the death of AKR-2B fibroblasts after serum removal, activity measurements were carried out to assess the activation of an YVAD- or DEVD-cleaving protease. The cleaving activity was measured using the fluorogenic substrates YVAD-amc and DEVD-amc.

In a recent publication [42] it was demonstrated that a YVAD-amc cleaving activity was rapidly stimulated (15 min) after initiating a cell death program by treating the cells with an anti-Fas antibody [43]. We have therefore measured this activity over a period of 60 min after serum removal. As shown in Fig. 6A there was no significant increase in YVAD-amc cleaving activity over this period. Any basal activity measured in this assay was not reduced by the inhibitor YVAD-cmk, so it has to be considered as nonspecific activity.

The results in Fig. 6A suggest that caspase 1 is not involved in death of AKR-2B fibroblasts after serum removal. In order to clarify, if caspase 1 isoforms are expressed and/or cleaved in the cells before and after serum removal, Western blot analyses with whole cell lysates were carried out using an antibody which recognizes the uncleaved caspase-1 isoforms as well as their fragments of 20 kDa. Figure 6C delineates that at least one isoform is expressed constitutively in AKR-2B cells. The apparent molecular weight of 42 kDa corresponding to the most heavily stained band leads to the conclusion that it is caspase 1a. During the time course of starvation after serum removal, there was no processing of the protein into its active subunits (20 kDa).

Unlike YVAD-amc, DEVD-amc cleavage increased drastically after serum removal (Fig. 6A). A maximum was reached 3 h after serum removal. This cleaving activity was completely blocked in the presence of 250 nM DEVD-cho. There was no basal activity in cytosolic extracts from control AKR-2B cells. These results indicate that a protease of the CPP32 family might be involved in cell death after serum removal, but that the activation of this enzyme is mediated by a pathway that does not include the proteolytic processing of caspase-1.

Even though no YVADase activity was found in AKR-2B, YVAD-cmk acts as a potent inhibitor of AKR-2B cell death. As it was shown recently with extracts from HL-60 cells, that YVAD-cmk is able to inhibit DEVD-amc cleaving activity *in vitro* [44], we tried to inhibit DEVD-amc cleavage in the presence of increasing concentrations of YVAD-cmk in extracts from AKR-2B cells. The kinetics of these reactions are shown in Fig. 6B. After 5 min a concentration of 600 nM YVAD-cmk lead to a complete inhibition of the DEVDase activity. This inhibitor (150 nM) was sufficient to block the reaction after 20 min. These results show that YVAD-cmk inhibits the DEVD-amc cleaving activity with high affinity at a quite fast rate. It is therefore likely that when applied to intact cells YVAD-cmk inhibits cell death after serum removal by blocking the activity of a DEVD cleaving enzyme.

DEVD-amc Cleavage Can Be Inhibited by Cell Protecting Reagents

As shown before, the time course for the activation of DEVD cleaving proteases correlates with that of cell disintegration. To further demonstrate an important role of these proteases in the process of cell disintegration, the influence of cell protecting reagents on the activity of DEVD-amc cleaving caspases was investigated. Figure 7 shows that all of these reagents are able to inhibit proteolytic activity of caspases belonging to the CPP32 family. Since these reagents act via different signal transduction pathways it can be concluded that all these pathways converge to one signal cascade preventing apoptosis upstream from the CPP32-like protease in AKR-2B cells.

Effect of Colchicine on the Early Loss of Actin Fibers after Serum Removal

The earliest macroscopic event after serum deprivation is the appearance of membrane blebbing. Using time lapsed video microscopy we observed that this blebbing is a dynamic process [27]. These morphological changes could be detected over a time period of 80 min after which the cells ceased to move and were therefore considered as dead. Early breakdown of actin fibers and/or loss of cell-matrix contact has been reported to be a prerequisite for cell shape alteration and at least for some cell lines an implication of these processes has been postulated in the cell death [8, 45–48]. The role of actin fibers breakdown was therefore investigated using AKR-2B cells. As shown in Fig. 8, there was a rapid breakdown of the cytoskeleton after serum removal. PDGF-BB did not prevent this breakdown but lead to a remodeling of the cytoskeleton which could be detected after 6 h. Surprisingly the addition of colchicine prevented the initial breakdown of the actin fibers. After 6 h a shortening of the fibers is detectable.

DISCUSSION

In a previous article the basic features of the death process of AKR-2B cells after serum deprivation were described [27]. In brief, cells round up after ~90 min, showing membrane blebs with berry-like structure. Cell surface changed continuously during the following 80 min after which time no further motion was detected and the cell was considered dead. Membrane leakiness was observed already 60 min after serum

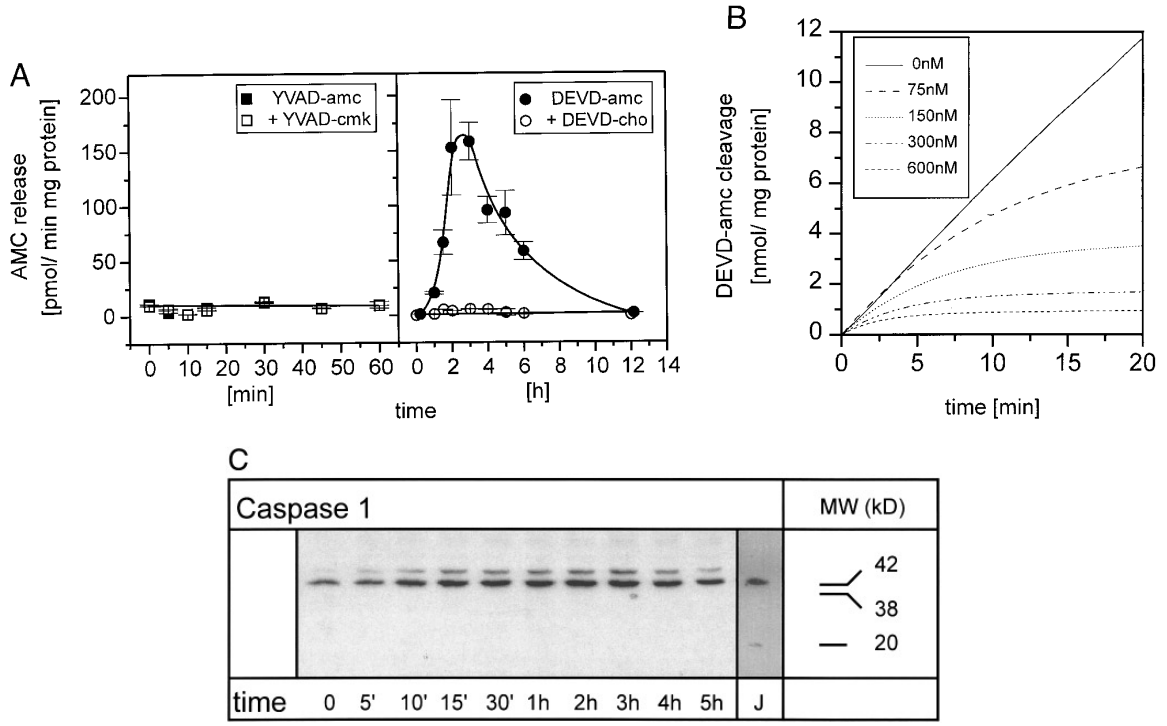

FIG. 6. (A) Determination of caspase activities in AKR-2B cells after serum removal AKR-2B cells were grown as described and starved for indicated times. To determine the cleaving activities, 50 μg protein of cytosolic extract were incubated with the specific fluorogenic substrates YVAD-amc (-■-) or DEVD-amc (-●-). Background activities were ascertained by adding 250 nM of the specific inhibitors to the reaction mixtures (YVAD-cmk: -□-) or DEVD-cho (-○-). (B) Effect of YVAD-cmk on DEVD-amc cleavage activity. 3 h after serum removal cytosolic extracts of AKR-2B cells were prepared and incubated with indicated concentrations of the inhibitor YVAD-cmk 1 min before the addition of the fluorogenic substrate DEVD-amc (final concentration 15 μM). The release of amc was monitored continuously. (C) Immunoblot analysis of caspase 1 in AKR-2B cells after serum deprivation AKR-2B cells were grown as described and cultured in serum-free MCDB 402 for indicated time. Whole cell lysates were subjected to a 12% SDS–PAGE followed by immunoblotting with antibodies that recognize the caspase 1 precursor (42 kDa) and the p20 subunit of the active caspase (cf. materials). The position of the molecular weight markers is indicated on the right. As a positive control a lysate from Jurkat cells which are known to express this caspase is shown on the right (J) (66).

removal and seemed to parallel nuclear condensation. At that time mitochondria are swollen and damaged. At this point "apoptotic" events stopped, since no DNA fragmentation occurred and no ingestion of the dead cell by its neighbors was observed. Though not all criteria for apoptosis are seen with AKR-2B cells, it is clear that these cells do not die via a simple necrotic pathway. Rather, the process of cell death includes both apoptotic and necrotic events. Especially, we have shown that these cells exhibit an extremely high energy state of 0.9 with an intracellular ATP-concentration of 6 mM, thus excluding the possibility that a drop in ATP content might lead to a destabilization of the cytoskeleton rendering the cells more fragile. Moreover, an ICE-like protease seems to be involved in the mechanism of death, suggesting an active cellular process rather than a suffering of the cells from unappropriate culture conditions. There is a growing list of protein members apparently involved in the regulation/activation of ICE-like proteases and the interaction of many of these components is being elucidated [2, 4, 6, 10, 11, 20, 21, 49–59].

Three temporal stages during apoptosis have been suggested recently by Newmeyer *et al.* [52]. The first of these, "initiation," would refer to events that cause entry into a common death pathway. The second stage, which was termed "sentencing" encompasses the intracellular events that commit the cell irreversibly to the cell death process. Finally the cell enters the "execution" stage, in which effector molecules, such as particular nucleases and proteases, accomplish the overt changes associated with apoptotic cells death.

The duration of the "initiation" phase is usually 60–90 min after the addition of an apoptotic stimulus. In several instances an activation of ICE-like proteases (caspase-1) is observed in the first phase [42, 60]. Also ceramides are reported to play a role in this phase [61]. The second phase (2–4 h) is characterized by the activation of CPP32-like proteases, the induction of permeability transitions (PT) in mitochondria, release of cyto-

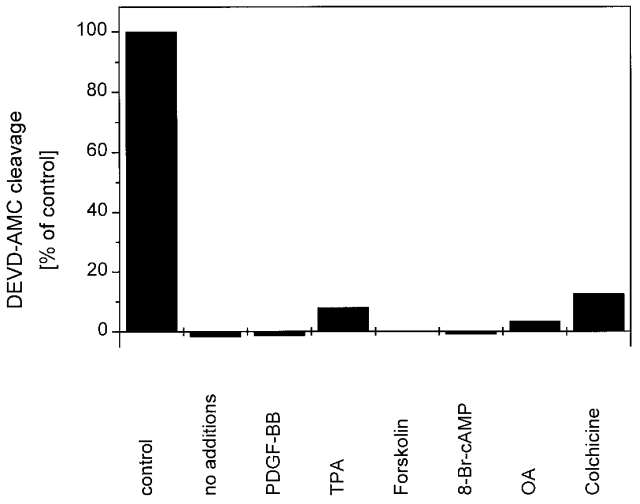

FIG. 7. Effect of protecting agents on DEVD-amc cleavage activity. Following serum removal, cells were incubated with different amounts of protecting agents as described in the legend to Fig. 2. The concentration of okadaic acid was 100 nM, and that of colchicine was 10 μM. After 3 h incubation cytosolic extracts were prepared and DEVD-amc cleavage activity was determined.

chrome c and a caspase activation factor from mitochondria, and the action of the apoptotic regulator protein Bcl-2. The activation of CPP32-like caspase has been observed in almost all investigated cells and seems to be a hallmark for the manifestation of apoptosis [42, 44, 51]. Based on the different kinetics of activation of caspase-1 and caspase-3, a sequence of activation was delineated. However, a putative direct caspase activation pathway fails to explain important facts such as latency between caspase-1 (15 min) and caspase-3 (2–4 h) activation, mitochondrial changes, and nuclear condensation.

The third phase, "execution," comprises events such as fodrin cleavage, lamin A, B, or C cleavage, and nuclear condensation.

We have recently characterized several events following serum removal in AKR-2B cells. Obviously a first latent phase exists, since the onset of massive cells death occurs after 90 min. During this stage the addition of "survival factors" suppresses the ongoing of apoptosis. However, we were unable to detect the activation of a YVADase (caspase-1) neither by using a fluorogenic substrate nor by Western blotting using an antiserum against caspase-1 (ICE). Clearly our data do not rule out the possible existence of an activated protease with a different substrate specificity. Nevertheless, our data are in agreement with recent findings that CPP32 (caspase-3)- and Mch2 (caspase-6)-like proteases are the major active caspases in apoptotic cells [42, 44, 51]. Importantly, in AKR-2B cells, in all instances the DEVDase activity is inhibited by substances that suppress cell death.

Cell death "execution" in AKR-2B cells is uncommon, since it lacks DNA-fragmentation. Clearly mitochondria are swollen and damaged in dead cells, but at least for a significant number of cells, the plasma membrane was permeable prior to a breakdown of the mitochondrial potential [27]. It seems that the timing of the events is somewhat deregulated leading to a premature damage of the plasma membrane. Upon energy deprivation due to cell leakage subsequent steps requiring ATP are probably blocked.

The present work describes the different signaling pathways involved in the protection of AKR-2B cells against cell death. Besides PDGF-BB and okadaic acid, which have been analyzed previously [26], forskolin, 8-Br-cAMP, colchicine, and TPA were effective survival reagents. PDGF-BB was the most potent factor, yielding complete protection over the long time period of 1 day. The other substances allowed full protection only for a shorter time of 6 h. The factors differed dramatically in their requirement for protein synthesis. In the presence of the protein synthesis inhibitor CHx only forskolin and partially 8-Br-cAMP were still protective. For the protective effects of PDGF-BB or TPA, respectively, protein biosynthesis was required. These results indicate that activation of cAMP-dependent signaling pathways leads to cell protection without the requirement of protein biosynthesis, whereas PKC-dependent pathways are dependent on protein synthesis. Though there were many similarities with Balb/c-3T3 cells, there are distinct implications for PDGF-BB-related signaling pathways in the suppression of cell death in AKR-2B cells. Importantly, activation of cAMP-dependent pathways by PDGF-BB plays a major role in Balb/-3T3 cells. This pathway has been elucidated recently: PDGF-receptor activation leads to an activation of the MAP-kinases which phosphorylate and thus activate the PLA_2, finally leading to increased synthesis of prostaglandins. These compounds then evoke an increase in the intracellular cAMP content [37]. In AKR-2B cells PDGF-BB is unable to activate cAMP-dependent pathways.

This discrepancy is not surprising since the effects of PDGF-BB on the two cell lines regarding the stimulation of cell division are quite distinct. More than 95% of AKR-2B cells divide after the administration of PDGF-AB or -BB, respectively, without further supplements like, e.g., insulin or IGF-I [30]. For Balbc/-3T3 cells, PDGF establishes a competence state and these cells require the continuous presence of insulin or IGF-I for progression through the cell cycle [62]. The reason for the different tuning might be a different availability of binding proteins containing SH2- or PTB-domains and thus a different recruitment in the "receptosomes."

Several growth factors originally characterized for their ability to induce proliferation have been shown to be potent regulators of cell survival and for some

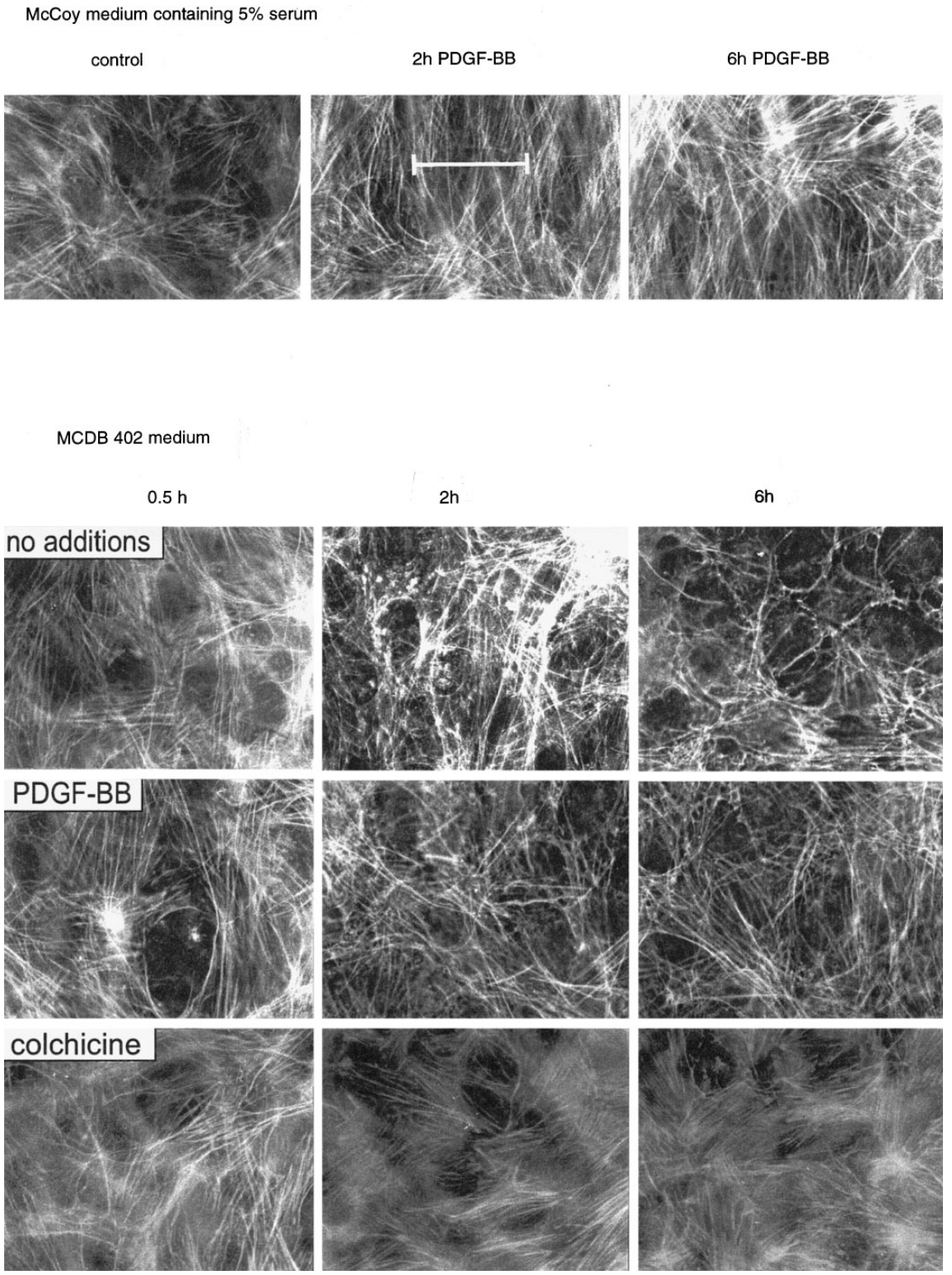

FIG. 8. Changes in the cytoskeleton of AKR-2B cells. Upper part: for control, AKR-2B cells were kept in McCoy medium. The left picture was taken at time zero (without addition of factors). As indicated PDGF-BB (50 ng/ml) was added for 2 or 6 h. For all other experiments the McCoy medium was replaced by the MDCB 402 medium without serum. The indicated factors were added for 0.5, 2, or 6 h. (PDGF-BB, 50 ng/ml or colchicine, 10 μM). The cells were then fixed in 3% paraformaldehyde and the actin was stained with FITC-labeled phalloidin. The bar represents 20 μm. Magnification was 630-fold.

cells, growth factor activated intracellular pathways were shown to be implicated in the cell protection. Upregulation of c-myc expression was shown to be involved in the apoptosis of NIH-3T3 cells which could be suppressed by ras signaling through PI(3)-kinase and PKB [63]. In PC-12 cells, too, PI(3)-kinase and PKB activation prevented apoptosis [64, 65]. As previously shown, in AKR-2B cells c-myc expression is not elevated [27]. Furthermore in these cells, death is not induced in the presence of PDGF-BB by the addition of wortmannin, a putative selective inhibitor of PI(3)-kinase (J. Hoppe, unpublished), dismissing an involvement of these components in the protection after PDGF-BB stimulation. Another connection between proliferation signals and apoptotic pathways is just emerging. First, the antiapoptotic regulator protein Bcl-2 targets the raf-1 kinase to mitochondria [20]. But so far the functional implications of this subcellular translocation are unknown. Second, the regulator of Bcl-2, Bad, is targeted by hyperphosphorylation to 14-3-3 proteins located at the cytoplasmatic membrane and is thus removed from Bcl-2 which then is able to effect protection [21]. But so far the kinase(s) involved remain to be identified.

We have shown that in AKR-2B cells after serum deprivation the activation of a caspase-3-like protease is prevented by addition of protective reagents that presumably act via different intracellular pathways: cAMP-dependent-kinases (forskolin and 8-Br-cAMP) or protein kinase C (TPA). Surprisingly, colchicine, an inhibitor of tubulin polymerization, which presumably does not use either of the above pathways, effectively suppressed cell death and activation of a caspase-3 like activity. As shown in Fig. 8 the addition of colchicine prevented the initial degradation of actin fibers raising the possibility that loss of cell contact and/or breakdown of the actin skeleton provides an initiation signal for cell death. The strict correlation between effecting survival and suppression of caspase activation, places the action of the cell protection reagents upstream of the DEVDase (caspase-3). The precise point of intervention remains to be identified. It would be interesting to see whether phosphorylation of Bad is common to all pathways.

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