Induction of Apoptosis by Protein Kinase Cδ Is Independent of Its Kinase Activity*

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Protein kinase C (PKC) is a family of phospholipid-dependent serine/threonine kinases comprising 10 isoforms that differ in their structure of up to four variable and three constant regions and their function (1, 2). These PKC isoforms are grouped into three classes: the “conventional” εPKCs PKC-α, -β (−βI and −βII), and -γ, which can be activated by diacylglycerol, phosphatidylinositol 4,5-bisphosphate, or phorbol esters and have a Ca2+-binding site in their second constant region, C2. The “novel” PKCs PKC-δ, -ε, -η, and -θ lack the C2 region and thus are Ca2+-independent but still diacylglycerol-, phosphatidylinositol-, and phorbol ester-responsive, whereas the “atypical” PKCs PKC-ζ and -λ/ι also lack the C2 region and are therefore Ca2+-independent and only respond to phosphatidylinositol 4,5-bisphosphate and not to diacylglycerol or phorbol esters (3, 4). Among other isoforms, PKCs have been found to be involved in intracellular signal transduction pathways that regulate growth and lead to differentiation and apoptosis (5–7). The biochemical mechanism of PKC activation has been elucidated following the discoveries of phorbol ester/diacylglycerol activation and phosphorylation of conventional PKCs on the activation loop by the PKC-activating phosphoinositide-dependent kinase (PDK1). This initial phosphorylation triggers the autophosphorylation at carboxy-terminal sites and thus the first step in order to generate the mature enzyme (8). However, the precise functions of all of the PKC isoforms are still largely unknown, and the role of PKCs in several important biological processes remains to be elucidated. Moreover, some reports about the function of PKCs are contradictory (9, 10). This is most likely due to the expression of different PKC isozyme subsets in the cellular systems investigated. Structurally, the PKC isoforms are quite similar, and also their in vitro substrate specificity is nearly identical (11). Nevertheless, the isoforms fulfill distinct and different biological functions in vivo. For example, PKCδ reduces the proliferation of NIH 3T3 fibroblasts and arrests Chinese hamster ovary cells in the G2/M phase in response to 12-O-tetradecanoylphorbol-13-acetate (TPA) (12) and induces differentiation of mouse promyeloid cells (6). In contrast, overexpression of PKCe in rodent fibroblasts stimulates cell proliferation, induces anchorage-independent growth, and causes tumors in nude mice (13, 14). Additionally, PKCδ has been implicated in apoptosis-mediating pathways for several years (3, 15, 16), but again, there is little consensus on its role. Apoptosis or programmed cell death is a major form of cell death characterized by well-defined morphological changes like condensation and fragmentation of nuclear chromatin, membrane blebbing, and formation of apoptotic bodies. Among the biochemical mediators of apoptosis, particularly important is the participation of caspases, a family of cysteine-dependent, aspartate-specific proteases (17–22). There are two different apoptotic pathways recognized to date. One is triggered by the ligation of transmembrane death receptors belonging to the tumor necrosis factor R1 family by specific ligands, transmitting a death signal that culminates in classic apoptotic cell death (23, 24). Upon binding of their ligands, death receptors directly recruit and activate caspase-8. A second apoptotic pathway includes a mitochondrial amplification loop. The mitochondrial contribution is regulated by members of the Bcl-2/Bcl-x family acting on the mitochondrial membrane potential. After depolarization of the membrane potential in the course of an apoptotic signaling cascade, cytochrome c and/or apoptosis-inducing factor (AIF) is released from the mitochondria (25). AIF is localized at the intermembrane space and co-localizes with hsp60, which remains in the matrix, after AIF is released (26). AIF has re-

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cently been characterized as a novel flavoprotein with striking homology to bacterial and plant oxidoreductases (27). Upon induction of apoptosis, AIF translocates from the mitochondria to the nucleus in a caspase-independent fashion, which induces generation of large DNA fragments (~50 kb) and chromatin condensation that resembles stage I nuclear apoptosis (26). Cytochrome c, after release into the cytoplasm, binds to Apaf-1 to form a complex that activates caspase-9 as the most apical caspase in the following cascade of proteolytic modifications (28). The first downstream target of caspase-9 is procaspase-3 (28, 29). Caspase-3 is indirectly regulated by members of the Bcl-2/Bcl-x family. Phorbol esters, and hence PKCs, have been shown to induce apoptosis in some cells and inhibit it in others (30, 31). Thus, the cell type appears to contribute to the results of phorbol ester treatment, most likely due to the varying spectrum of PKC isoforms expressed in different cells. When the contributions of activations of individual PKC isoforms were studied, it was generally agreed that PKCδ is proapoptotic in function. Indeed, overexpression of PKCδ in keratinocytes and in prostate cancer cells led to TPA-dependent induction of apoptosis (32, 33). Several studies have shown PKCδ to be cleaved by caspase-3 in its third variable region, V3, to a catalytically active 40-kDa fragment in cells committed to apoptosis (32, 33). PKCδ has also been linked to the activation of caspase-3, leading to the presumption that PKCδ may play a role in apoptosis both upstream and downstream of the caspase cascade (36). PKCδ has been shown to translocate partly to the mitochondria upon stimulation of keratinocytes and U-937 leukemic cells with the phorbol ester TPA. This was accompanied by alteration of the mitochondrial membrane potential (32) and release of cytochrome c (30).

These data suggested that PKCδ plays a major role in apoptosis via the mitochondrial amplification loop in several different cell types. Nonetheless, there are reports that suggest that PKCδ can inhibit apoptosis in other cells (31). PKCe, in contrast, has been described as a predominantly antiapoptotic isozyme (37).

Utilizing GFP-PKC expression vectors to examine PKCδ-induced apoptosis, we compared the potential of PKCδ and PKCe, the two most divergent enzymes, to induce apoptosis in the rat aortic smooth muscle cell line A7r5. In addition, we investigated whether the kinase activity of PKCδ was essential to exert its apoptotic effect.

EXPERIMENTAL PROCEDURES

Plasmids—The GFP expression vectors have been described elsewhere (38). As control, carboxyl-terminal fusion proteins of PKCδ, kinase-negative PKCδ (PKCδK376R) (5), and PKCe were generated by cloning the respective mouse cDNAs in pE-GFP-C1 mammalian expression vectors. The other PKC expression vectors have been described previously (6).

Cell Culture and Transfections—The vascular smooth muscle cell line A7r5 was used in all experiments. Cells were grown in Dulbecco’s modified Eagle’s medium without Phenol Red containing 10% fetal calf serum and glutamine in uncoated 80-cm² culture flasks from Nunc or in six-well cell culture plates. The medium was changed every 3 days. A7r5 cells were seeded freshly, and transfections were performed after about 24 h of culture or when cells were grown to 70% confluence. For transient transfections, 2 μg of the respective plasmid DNA was dissolved in the above medium without serum. Superfect Transfection Reagent (Qiagen) was added according to the manufacturer’s recommendations. Transfected cells were examined 2 days after transfection by confocal laser-scanning microscopy or by fluorescence microscopy.

TPA was used at 0.5 μM, dithiothreitol at 10 μM, and the PKC inhibitor GF109203X at 1 and 3 μM.

Cell Lysis and Kinase Assay—Cells were pelleted at 400 × g and washed once with PBS. The pellets were resuspended in lysis buffer (39) consisting of Tris-buffered saline with 2 mM EDTA and EGTA each, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1% octyl glycoside. Additionally, cells were briefly sonicated in this buffer on ice, with three pulses of 5 s. This lysate was centrifuged at 30,000 × g for 10 min. The supernatant was then used for Western blot analysis or was incubated with rabbit anti-GFP antibodies (1:2500; Invitrogen) overnight for immunoprecipitation. The immunoprecipitate was consecutively washed in protein binding buffer and in a buffer containing protein A-Sepharose beads (Amersham BioSciences) and incubating the solution for 1 h. The beads were collected by centrifugation at 400 × g and washed twice in ice-cold kinase assay buffer, consisting of 10 mM Tris·HCl, pH 7.8, 0.1 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂, 0.5 mM EDTA and EGTA, 1 mM dithiothreitol, 0.01% Triton X-100. To validate that the kinase-negative mutant of PKCδ does not phosphorylate substrates, cells were transiently transfected either with GFP-PKCδ(Δ) or the kinase-dead mutant GFP-PKCδK376R. A confluent layer of each cell type from 10-cm culture dishes was homogenized in 500 μl of lysis buffer. GFP-PKCδ was isolated by immunoprecipitation. The kinase assay was performed in 10 μl of kinase assay buffer, 2 μl each of TPA and phosphatidyserine from stock solutions of 1 μg/ml were added, and 3 μl of 1 mg/ml of a basic protein were added as substrate for the phosphorylation. 10 μM ATP and 10 μCi of [γ-32P]ATP were added immediately, and the reaction proceeded for 10 min. A positive control contained purified PKCδ instead of the beads, and in a negative control the assay was void of PKC. The reaction was stopped by adding an equal volume of Laemmli SDS-PAGE sample buffer. Aliquots of the mixture were then subjected to SDS-PAGE and exposed to a photographic film for about 30 min.

Chromatin Condensation—Cells were treated with 0.5 μM TPA for 5 h, and DNA condensation was visualized after DNA staining with 0.05 mg/ml Hoechst 33342 30 min prior to collecting the cells. Cells were scraped off and spun down at 400 × g and washed once with PBS. Cells were resuspended in 5% Mowiol 40–88 (Aldrich) in PBS. A drop of 10 μl was spread on a glass microscope slide and covered with a coverslip. Cells were examined under a fluorescence microscope. Green fluorescent cells (80–100 each per experiment) were chosen at random, and their nuclei were observed under blue fluorescence. Cells with intact nuclei were counted as nonapoptotic, and cells with condensed DNA or completely disintegrated nuclei were counted as apoptotic.

Mitochondrial Staining—A7r5 cells were incubated with 10 μM Calcein-AM and Mitotracker Red (Molecular Probes, Inc., Eugene, OR) for 30 min at 37 °C. The cells were washed once with medium and examined by laser confocal microscopy.

Preparation of Mitochondria—A7r5 cells were suspended in 0.25 M sucrose, 20 mM Tris, 1 mM EDTA to yield a thick suspension of cells in 1 ml of buffer. Cells were then centrifuged at 10,000 g for 7 min. The pellet was then treated as described above. The supernatants were then combined and centrifuged at 10,000 × g for 10 min. The responding mitochondria pellet was taken up in the above described sucrose buffer in an appropriate volume.

Annexin Staining of Apoptotic Cells—Transiently GFP-PKCδ-transfected A7r5 cells were incubated with 5 μM annexin V-Cy3 (Molecular Probes, Inc.) and washed twice in annexin binding buffer. After induction of apoptosis, cells were washed with cold PBS. Washed cells were then suspended in annexin binding buffer, containing 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, at pH 7.4. To a 100-μl cell suspension, 15 μl of an annexin-V-Cy3.18 conjugate solution were added to a final concentration of 3.75 μg/ml. Cells were incubated for 15 min at room temperature. After incubation, cells were washed twice in annexin binding buffer. Aliquots of this cell suspension were analyzed under a fluorescence microscope at the appropriate wavelength.

Immunohistochemistry—A7r5 cells were grown on glass coverslips in the wells of a six-well plate. Cells were transiently transfected with GFP-PKCδ, and after 36 h zVAD.fmk was added to a final concentration of 1 μM. After 15 min of incubation, 1 μM of dithiothreitol in a medium containing 10 μM TPA was added in order to induce apoptosis via PKCδ. After incubation, cells were washed in PBS and then fixed with 4% paraformaldehyde in 4% sucrose. After 30 min of fixation at room temperature, the cells were washed twice with PBS. Excess formaldehyde was then neutralized with 4% glycine in 4% sucrose, and after another 30 min of neutralization the cells were thoroughly washed with PBS. A solution of 0.5% saponin in PBS was made 10% with goat serum. To 300 μl of this
solution, 6 μl of a polyclonal rabbit antibody against cleaved caspase-3 (Cleaved Caspase-3 (Asp-175) Antibody; Cell Signaling, Beverly, MA) was added to yield a dilution of 1:50. Fixed cells were incubated in this reagent for 1 h and then washed three times in PBS. Cells were then treated with a secondary donkey anti-rabbit CyTM3 IgG at a dilution of 1:500 in 0.5% saponin in PBS for 1 h. After washing 3 times with PBS, labeled cleaved caspase-3 was detected by fluorescence microscopy.

RESULTS

Translocation of PKCδ Induces Apoptosis in A7r5 Cells—We utilized the rat aortic smooth muscle cell line A7r5 to investigate the role of PKCδ in apoptosis. The cells were transiently transfected with GFP-tagged PKCδ and PKCe or with GFP-PKCβ (as a control) as well as with GFP alone. To demonstrate that only stimulation with TPA provoked the apoptotic effect and not the presence of PKCs alone, cells overexpressing PKCδ, PKCe, or PKCβ, were examined without TPA being added to the medium. As expected, no significant effect from any of the overexpressed enzymes could be observed in unstimulated cells. Treatment of untransfected cells or cells expressing GFP alone with 0.5 μM TPA did not result in any significant morphological changes. Also, cells transfected with GFP-PKCe or -PKCβ, did not show any sign of apoptosis. In contrast, the GFP-PKCδ overexpressers rounded up within 1 h of treatment with TPA or 1,2-dioctanoyl-sn-glycerol, a membrane-permeable derivative of the endogenous PKC activator diacylglycerol (data not shown). Subsequently, cells detached from the culture dish surface, suggesting incipient apoptosis. These initial experiments provided the basis for our further investigations.

As shown in detail in Fig. 1A, TPA stimulation of GFP-PKCδ induced massive membrane blebbing, a more definitive sign of apoptosis, as early as 30 min after TPA application. In comparison, no sign of membrane blebbing could be observed in the GFP-PKCε- or GFP-PKCβ-overexpressing cells. To confirm the degree of overexpression of the GFP fusion proteins, transfected or untransfected cells were lysed, and the lysates were subjected to Western blot analysis of GFP and the respective PKC isozymes after SDS-PAGE with chemiluminescence to detect the respective bands. As is evident from Fig. 1B, transfected cell populations exerted a markedly increased band intensity for the respective isozymes, compared with untransfected cell populations. Also, the levels of overexpression of GFP-PKCδ and GFP-PKCe are comparable.

The most prominent effect of TPA is to induce a translocation of affected PKCs. Laser-scanning confocal microscopy showed GFP-PKC fusion proteins translocating to various membrane compartments, following TPA application. Collecting images at different time points offered the possibility of following the translocation at distinct stages. As shown in Fig. 2A, PKCδ was predominantly located diffusely in the cytoplasm in unstimulated cells. Upon TPA stimulation, the enzyme rapidly trans-
located to the plasma membrane and nuclear envelope. Subsequently, the enzyme clustered at distinct regions both at the membranes and within the cytoplasm. As early as 20 min after stimulation, the PKC clustering was followed by the appearance of membrane blebs, an early unambiguous hallmark of apoptosis. After 60 min, the cells showed multiple large membrane blebs and started to detach from the surface. GFP-PKC/\(H9254\)-transfected cells, in comparison, did not turn into apoptosis after stimulation with TPA. However, a slight translocation of PKC/\(H9254\) can be observed as well (Fig. 2, A and B). Fig. 2B shows single images of a stack of pictures taken along the z axis of a GFP-PKC/\(H9254\)-overexpressing cell that had been treated with TPA for 1 h prior to the examination, whereas PKC/\(H9254\)-transfected cells detached from the surface and contracted PKC/\(H9254\)-transfected cells did not alter their morphology.

To further confirm the presumption that cells overexpressing GFP-PKC\(\delta\) become apoptotic after TPA stimulation, chromatin condensation, an additional characteristic marker of apoptosis, was visualized by staining the nuclei with the blue fluorescing FIG. 3. A, A7r5 cells were transiently transfected with GFP-PKC\(\delta\). The cells were treated with 0.5 \(\mu\)M TPA for 5 h. The nuclei were visualized by incubating the cells with the DNA staining dye Hoechst 33342 at 0.5 \(\mu\)g/ml for 30 min prior to analysis in a fluorescence microscope. Stained nuclei appear blue fluorescing as shown in the right image. The red arrows indicate the position of the green fluorescing GFP-PKC\(\delta\)-overexpressing cells in the left image and show the same cells in the right image. Only the PKC\(\delta\)-overexpressing cells showed chromatin condensation, whereas the nontransfected cells contained intact nuclei. B, a GFP-PKC\(\delta\)-overexpressing cell after 3 h of TPA stimulation was selected to confirm apoptosis as the cause of cell death by staining with annexin-V. The nucleus clearly shows condensed DNA, and additionally the cell shows the typical ring of red fluorescing annexin bound on the cell surface, an unambiguous sign of apoptosis.

PKC\(\delta\)-induced Apoptosis Is Independent of Kinase Activity

Fig. 4. a, stimulation of PKC\(\delta\) leads to depolarization of the mitochondrial membrane. Cells were stained with MitoTracker Red CMXROS for 20 min immediately before PKC stimulation. Initially, all cells showed active mitochondria (in red) irrespective of PKC\(\delta\) overexpression. After 1 h, the red fluorescence of the mitochondria was markedly decreased compared with the green fluorescence of the GFP-PKC\(\delta\). b, a complete loss of mitochondrial membrane potential was observed in some cells as early as 45 min after PKC\(\delta\) stimulation with TPA. The left panel images show the green fluorescing PKC\(\delta\)-overexpressing cells, and the right panel images show the red fluorescing mitochondria of the respective fields. After cells had been stimulated with TPA for 45 min, virtually no active mitochondria were detectable in the GFP-PKC\(\delta\)-overexpressing cell. The white frame in the lower right panel encircles the area of the cell that lost its membrane potential and hence is not stained by MitoTracker. c, mitochondrial fractions of GFP-PKC\(\delta\)-transfected cells were prepared, and homogenates were subjected to Western blot analysis with antibodies specific against GFP. Prior to homogenization, cells were treated with TPA for different periods of time as indicated in minutes.
DNA dye Hoechst 33342 at defined time points after the induction of apoptosis by TPA. Chromatin condensation was detected as early as 3 h after PKC stimulation. After 5 h of PKC stimulation, all GFP-PKCα-overexpressing cells revealed condensed or even completely disintegrated nuclei (Fig. 3A). After induction of apoptosis by TPA, cells were also counterstained with annexin-V-Cy3.18 in order to confirm apoptosis as the cause of cell death besides chromatin condensation and membrane blebbing. As is evident from Fig. 3B, GFP-PKCα-overexpressing cells stained positive with annexin as well. To prove that GFP alone or GFP-PKCα did not exert any additional apoptotic effect, A7r5 cells were transfected with these expression vectors and examined for chromatin condensation after TPA stimulation. Green fluorescing cells that overexpressed either of these proteins showed only a marginal number of apoptotic cells (<10%), not significantly different from untransfected cells. Nevertheless, intracellular PKCε translocation in the respective cells could be seen after TPA stimulation, indicating that this isozyme was, indeed, activated, but stimulation did not lead to apoptosis.

PKCα-induced Apoptosis Is Independent of Kinase Activity

Fig. 5. I (green), cells were transfected with GFP-PKCα, 30 min prior to TPA stimulation, cells were treated with zVAD.fmk (+zVAD) or left without the caspase inhibitor (−zVAD). II (blue), the cells were then incubated with phorbol ester for 3 h, and the nuclei were visualized by adding Hoechst 33342. III (red), finally, the cells were stained against cleaved caspase-3 with a CyTM3-tagged antibody. Both GFP-PKCα transfected cells treated with and without zVAD prior to a 3-h period of TPA stimulation showed shrunk nuclei with condensed chromatin as a sign of apoptosis. b, in control experiments, apoptosis was induced by treatment with β-ketocholesterol. Cells were either incubated with zVAD.fmk (+zVAD; B) previous to the induction of apoptosis or left without this caspase inhibitor (−zVAD; A). Without zVAD.fmk, cells became apoptotic, as is indicated by the condensed nucleus in the upper image of A. The same cell (red arrows) also stained positive for cleaved caspase-3. In the upper image of B, nuclei appear unchanged after incubation with β-ketocholesterol in the presence of zVAD. No staining with cleaved caspase-3 antibody could be detected.
PKCδ-induced apoptosis is independent of kinase activity

A7r5 cells were transiently transfected with GFP-tagged wild type PKCδ (δ), kinase-negative PKCδ (δ-), pEGFP alone (GFP), or untagged PKCδ (pMV7δ). A7r5 cells that overexpressed Bcl-2 showed reduced rates of apoptosis after TPA treatment, whereas the caspase inhibitor zVAD.fmk revealed no significant effects. There were no detectable changes in untransfected cells when treated with TPA, GF109203X, or zVAD.fmk. The inset shows a Western blot analysis of Bcl-2 overexpression in the respective stable transfected cells versus untransfected control cells. Lane 1 represents the homogenate of untransfected cells, and lane 2 shows the result from Bcl-2-transfected cells. Homogenates were prepared from a confluent layer of cells grown in 10-cm culture dishes. Cells were scraped off in 500 μl of 2% SDS-PAGE sample buffer and homogenated in microreaction cups. An aliquot of 10 μl was used for the samples shown here.

A 1-h time course shows the gradual deprivation of the MitoTracker fluorescence in a transfected cell after TPA stimulation (Fig. 4A). Single cells were observed to lose their mitochondrial membrane potential already 20 min after the phorbol ester application (Fig. 4B). Although a direct involvement of the mitochondrion in this PKCδ-induced apoptosis appears to be obvious, a co-localization of the red fluorescing MitoTracker-labeled mitochondria and the green fluorescing GFP-PKCδ cannot be demonstrated. In a Western analysis, a slightly increased signal of PKCδ could be detected in mitochondrial fractions after 30 min of phorbol ester stimulation of cells (Fig. 4C). In these experiments, no PKCδ cleavage fragments were found (data not shown). Thus, although the majority of PKCδ molecules appeared to be located at the plasma membrane and the nuclear envelope, these data suggested that apoptosis was induced via the mitochondrial amplification loop. Mitochondria can contribute to apoptosis by cytochrome c release and activation of caspase-3 (41) or by release of AIF (42). As could be demonstrated here, green fluorescing and hence GFP-PKCδ-overexpressing cells induced apoptosis when activated with phorbol ester as was judged by the disintegration of the nuclei and chromatin condensation, respectively. Nevertheless, the stimulation of PKCδ with TPA was not followed by a cleavage of caspase-3, as is shown in Fig. 5A, and immunohistochemical staining against cleaved caspase-3 remained negative. Apoptosis could be seen in both cells that had been treated with zVAD.fmk previous to PKCδ stimulation and cells that were not treated with zVAD.fmk. The caspase inhibitor was active against apoptosis of cells that had been treated with β-ketocholésterol to induce apoptosis via the caspase cascade (Fig. 5B). Although our data as presented in Fig. 5 already suggested no caspase activation, we examined in more detail whether caspasess might be responsible for the apoptotic effect, and subsequently we incubated GFP-PKCδ-overexpressing cells with the membrane-permeable caspase inhibitor zVAD.fmk before TPA stimulation of GFP-PKCδ. Overexpressing cells of several different cell preparations were tested for chromatin condensation and were statistically analyzed. However, again, zVAD.fmk could not significantly prevent apoptosis of PKCδ-overexpressing cells in response to TPA (Fig. 6). In parallel, homogenates of stimulated and unstimulated cells were fractionated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were probed against cleaved caspase-3 by the Western technique. In the course of 4 h, no detectable amounts of caspase-3 were visible (data not shown). As was recently described by Engedal et al. (43), in three other cell lines, zVAD.fmk was not able to prevent apoptosis either when cells were treated with TPA.

The above results suggest that PKCδ-induced apoptosis of A7r5 vascular smooth muscle cells occurs independently of immediate caspase activation. The redistribution of PKCs initially suggested that apoptosis might be induced by activation of membrane components (e.g. death receptors), whereas the loss of mitochondrial membrane potential rather suggested a pathway via the mitochondria amplification loop. To distinguish between these two, we examined the effects of Bel-2 on PKCδ-induced apoptosis. Bel-2 inhibits only the latter pathway and counteracts the release of cytochrome c and AIF from the intermembrane space of mitochondria upon apoptotic stimuli (44). We utilized a stable transfected line of A7r5 cells in which Bel-2 was overexpressed more than 10-fold, as judged by Western blot. As shown in Fig. 6, overexpression of Bel-2 inhibited the induction of apoptosis by more than 50% in GFP-PKCδ-overexpressing cells, confirming the involvement of in PKCδ-induced apoptosis. These data as well as the lack of DNA laddering (not shown) upon PKCδ-induced apoptosis indicate AIF release as one possible cause of apoptosis (42).

PKCδ Kinase Activity is Not Involved in Apoptosis Induction—If apoptosis occurred independently of caspase activity, it might have been possible that PKCδ exerted its apoptogenic effect without being cleaved by caspases to a constitutively active catalytic fragment, as was postulated earlier (28). Therefore, we examined whether the PKC kinase activity was essential to induce apoptosis, and we applied the PKC-specific membrane-permeable inhibitor GF109203X at 1 and 3 μM to cells...
overexpressing GFP-PKCδ before TPA was added. Both concentrations of the inhibitor reduced the PKC kinase activity to undetectable levels (Fig. 7) and, as expected, did not alter GFP-PKCδ translocation to membranes after TPA stimulation. Surprisingly, the induction of apoptosis was completely unaffected by the presence of the PKC inhibitor, suggesting that the kinase activity is, in fact, dispensable for this PKC function. In all experiments performed, PKC translocation preceded apoptosis. These findings suggested that PKCδ-induced apoptosis depends on the translocation of the enzyme but is independent of its kinase activity, revealing a new aspect of PKC signaling. To confirm this observation, we generated a kinase-negative GFP-PKCδ (–) mutant. As shown in Fig. 7, this protein is devoid of kinase activity. We examined its apoptosis-inducing potential by overexpressing it in A7r5 cells. Surprisingly, the GFP-PKCδ(–) also induced apoptosis after TPA treatment to a degree almost identical to that induced by the wild-type enzyme (Fig. 6). Analysis of the distribution of the kinase-negative GFP-PKCδ(–) revealed a pattern identical to that of the wild-type enzyme and again the appearance of blebs within 30 min (Fig. 8A). These data prove that the induction of apoptosis by PKCδ is independent of its kinase activity but depends on the translocation to distinct subcellular compartments.

To further substantiate our findings, we infected A7r5 cells with adenovirus that encoded GFP-PKCδ or GFP-PKCδ(–). As shown in Fig. 8, apoptosis could be induced by TPA treatment of cells that overexpressed either wild-type PKCδ or the GFP-PKCδ(–) mutant, whereas TPA-treated cells infected with wild-type adenovirus (Fig. 8, Adeno) showed no sign of apoptosis. These data confirm that it is not the kinase activity but rather the TPA-induced changes in subcellular compartmentalization of PKCδ that are important for apoptosis induction.

To eliminate possible artifacts caused by GFP-tagged proteins, we also examined the apoptotic potential of untagged PKCδ. A7r5 cells were cotransfected with the PKCδ expression vector pMV7δ along with pEGFP (as a marker of successfully transfected cells). The cells were stimulated with TPA and examined for apoptosis. Again, overexpression of PKCδ, irrespective of the kinase activity, resulted in TPA-dependent induction of apoptosis, whereas overexpressed, activated PKCε revealed no significant differences from untransfected cells (Fig. 3A).

DISCUSSION

Smooth muscle cells represent a cell type in which the role of PKC has been described as both pro- and antiapoptotic (10, 45). Hence, we utilized A7r5, a rat aortic smooth muscle cell line, to thoroughly investigate the role of different PKC isoforms in apoptosis. We focused on PKCδ and PKCε, since these two isozymes appear to have opposing biological functions. Cells were transfected with the respective GFP-PKC expression vectors and examined for signs of apoptosis. The data presented here identify PKCδ as a signal-regulated key component of an apoptosis-inducing pathway that can trigger the mitochondrial apoptosis machinery. A key finding is that this function of PKCδ depends primarily on its translocation and subcellular localization but not on the kinase activity. As is evident from the control experiments, PKCδ, but not the GFP tag, was responsible for the induction of apoptosis, as was established
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by employing untagged PKCδ or GFP alone. First, untagged PKCδ was able to induce apoptosis to the same extent as the GFP-tagged PKCδ. Second, neither GFP alone nor GFP-PKCδ could induce apoptosis. These experiments further showed that the induction of apoptosis was isozyme-specific, since GFP-PKCε (although this isozyme belongs to the same subgroup of the PKC family as PKCδ does) and also GFP-PKCβ1, as an exponent of the conventional isozymes did not induce apoptosis upon TPA stimulation. All three isozymes, however, are susceptible to phorbol ester stimulation. Therefore, it is obvious that the translocation-connected apoptotic effect is specific to PKCδ.

Since activated protein kinase B/Akt transmits survival signals generated by binding of growth factors to their respective cell surface receptors (47), we examined whether PKC overexpression might have influenced protein kinase B/Akt levels. We found that the overall amount of protein kinase B/Akt in PKCδ-overexpressing cells was slightly decreased compared with nontransfected cells. However, the levels of phosphorylated, and hence activated, protein kinase B/Akt were not significantly different in PKCδ-overexpressing or nontransfected cells.

Accumulation of PKCδ at mitochondria 20 min or later after TPA stimulation could not be shown by optical means, and an induced co-localization of PKCδ with mitochondria could not be seen. Western analysis of mitochondria-enriched fractions after different time points of TPA stimulation showed ambiguous results, and no significant enrichment of PKCδ in the mitochondrial fraction could be determined. Moreover, no cleaved PKCδ was detected in stimulated cells overexpressing this isozyme. Nevertheless, the involvement of mitochondria in PKCδ-induced apoptosis is indubitable. Both the apoptosis-decreasing effect of Bcl-2 in cells stable transfected with this anti-apoptotic protein and the loss of mitochondrial membrane potential upon TPA stimulation in cells transfected with PKCδ proved that the apoptotic effect of PKCδ occurs on the mitochondrial level. However, in the rat vascular smooth muscle cell line, A7r5, PKCδ obviously does not act directly on the mitochondrial level; rather, its effect is transmitted to the mitochondria through one or more PKCδ-binding proteins. Cleaved caspase-3 could not be detected after application of TPA, either in an immune histochemical assay or by Western analysis of cell lysates. Also, inhibition of caspases with the membrane-permeable caspase inhibitor zVAD.fmk did not prevent apoptosis. Therefore, it is obvious that not the release of cytochrome c, but rather the concomitant release of AIF or other caspase-independent reactions, appears to be the reason for the programmed cell death caused by the action of stimulated PKCδ. This assumption was further substantiated by the fact that DNA laddering, a typical sign of the caspase cascade, and hence activated, protein kinase B/Akt were not significantly decreased compared with nontransfected cells when stimulated with TPA. These experiments rule out effects due to the vector. However, there seems to be an effect due to the kinase activity. As can be seen in Fig. 6, the percentage of positive cells in experiments with kinase-deficient PKCδ mutants is slightly but significantly lower than in PKCδ-positive cells. However, the application of the PKC inhibitor increased the apoptosis rate in both cases. The data presented here (i.e., the induction of apoptosis by PKCδ independently of its kinase activity) are not in agreement with work published by other groups (32, 33). This discrepancy cannot be fully explained yet, but it may result from the different cell lines used. Since translocation seemed to be essential for PKCδ to induce apoptosis, we assume that this isozyme exerts its apoptogenic effect by binding to distinct intracellular structures, especially by interaction with target proteins, yet to be determined. This interaction may occur on a one-to-one basis and therefore necessitate larger amounts of PKCδ protein than intrinsically present in A7r5 cells. If the kinase activity were responsible for inducing apoptosis upon phorbol ester application, the low amount of intrinsic PKCδ would be sufficient to accomplish programmed cell death after TPA. This explains the fact that untransfected cells, which express only low levels of endogenous PKCδ, show no sign of apoptosis when stimulated with TPA. Also, PKCδ obviously does not directly act on the mitochondrial level, but it does affect the mitochondria apparently indirectly at such a rate that their membrane potential quickly depolarizes. Moreover, this supports our finding that the catalytic activity of PKCδ was not the origin of its apoptogenic effect, but this effect derived from its character as a signal-regulated binding/scaffolding protein. The target proteins for PKCδ that are responsible for the induction of apoptosis are most likely antiapoptotic proteins associated with the mitochondria, such as Bcl-2 family members. Our assumption is supported by the observation that mitochondrial membranes quickly depolarize when apoptosis is initiated by TPA and also that Bcl-2 is capable of decreasing the degree of PKCδ-induced apoptosis considerably. However, we have not yet determined the postulated PKCδ-associated protein(s). Our results reveal PKCδ as a multifunctional protein, whose biological role is not solely determined by its kinase activity but also by its function as capping or scavenging protein that binds to “antiapoptotic” target proteins.

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REFERENCES

1. Toker, A. (1998) Front. Biosci. 3, D1134–D1147
2. Parekh, D. B., Ziegler, W., and Parker, P. J. (2000) EMBO J. 19, 496–503
3. Dempsey, E. C., Newton, A. C., Moehly-Rosen, D., Fields, A. P., Reyland, M. E., Insel, P. A., and Messing, R. O. (2000) Am. J. Physiol. 279, L429–L438
4. Musashi, M., Ota, S., and Shiroshita, N. (2000) Int. J. Hematol. 72, 12–19
5. Li, W., Yu, J. C., Shin, D. Y., and Pierce, J. H. (1998) J. Biol. Chem. 273, 8311–8318
6. Mischak, H., Pierre, J. H., Goodnight, J., Kazanietz, M. G., Blumberg, P. M., and Mushinski, J. F. (1993) J. Biol. Chem. 268, 20110–20115
7. Assender, J. W., Kontny, E., and Fredholm, B. B. (1994) FEBS Lett. 342, 76–80
8. Davis, E. M., Toker, A., and Newton, A. C. (1998) Curr. Biol. 8, 1366–1375
9. Webb, P. R., Wang, K. Q., Scheel-Toellner, D., Pongracz, J., Salmon, M., and Lord, J. M. (2000) Apoptosis 5, 451–458
10. Wert, M. M., and Palfrey, H. C. (2000) Biochem. J. 352, 175–182
11. Kazanietz, M. G., Bustelo, X. R., Barbacid, M., Kolch, W., Mischak, H., Wong, G., Pettit, G. R., Bruns, J. D., and Blumberg, P. M. (1994) J. Biol. Chem. 269, 11590–11594
12. Watanabe, T., Ono, Y., Taniyama, Y., Hazama, K., Igarashi, K., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10159–10163
13. Mischak, H., Goodnight, J. A., Kolch, W., Martiny-Baron, G., Schaechtle, C., Kazanietz, M. G., Blumberg, P. M., Pierce, J. H., and Mushinski, J. F. (1993) J. Biol. Chem. 268, 6090–6096
14. Caece, A. M., Guadagno, S. N., Krauss, R. S., Fabbro, D., and Weinstein, I. B. (1993) Oncogene 8, 2095–2104
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15. Ghayur, T., Hugunin, M., Talanian, R. V., Ratnoff, S., Quinlan, C., Emoto, Y., Pandey, P., Datta, R., Huang, Y., Kharbanda, S., Allen, H., Kamen, R., Wong, W., and Kufe, D. (1996) J. Exp. Med. 184, 2399–2404
16. Cross, T. G., Scheel-Toellner, D., Henriquez, N. V., Deacon, E., Salmon, M., and Lord, J. M. (2000) Exp. Cell Res. 256, 34–41
17. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
18. Cohen, G. M. (1997) Cell 88, 17–19
19. Martin, S. J., and Green, D. R. (1995) Cell 80, 349–352
20. Cryns, V., and Yuan, J. (1998) Genes Dev. 12, 1551–1570
21. Thornberry, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
22. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Br. J. Cancer. 2399, 276, 1–16
23. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
24. Ware, C. F., Santee, S., and Glass, A. (eds) (1998) in The Cytokine Handbook M., Ghayur, T., Wong, W. W., Kamen, R., and Kroemer, G. (1995) Exp. Med. 192, 571–580
25. Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ravagnan, L., Saraiva, L. M., Haouzi, D., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. (2000) FASEB J. 14, 729–739
26. Daugas, E., Susin, S. A., Zamzami, N., Ferri, K. F., Irinopoulou, T., Larochette, N., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. (2000) FASEB J. 14, 729–739
27. Miramont, M. D., Costantini, P., Ravagnan, L., Saraiva, L. M., Haouzi, D., Prevost, M. C., Leber, B., Andrews, D., Penninger, J., and Kroemer, G. (2000) J. Exp. Med. 192, 571–580
28. Li, P., Nijhawan, D., Budhardo, I., Srinivasalu, S. M., Ahmad, M., Almemri, E. S., and Wang, X. (1997) Cell 91, 479–489
29. Srinivasalu, S. M., Ahmad, M., Fernandes-Almemri, T., and Almemri, E. S. (1998) Mol. Cell. 1, 949–957
30. Majumder, P. K., Pandey, P., Sun, X., Cheng, K., Datta, R., Saxena, S., Kharbanda, S., and Kufe, D. (2000) J. Biol. Chem. 275, 21793–21796
31. Romanova, L. Y., Alexandre, I. A., Schwab, G., Hilbert, D. M., Mushinski, J. F., and Nordan, R. P. (1996) Biochemistry 35, 9900–9906
32. Li, L., Lorenzo, P. S., Boge, K., Blumberg, P. M., and Yuspa, S. H. (1999) Mol. Cell. Biol. 19, 8547–8558
33. Fujii, T., Garcia-Bernejo, M. L., Bernabo, J. L., Caamaño, J., Ohba, M., Kuroki, T., Li, L., Yuspa, S. H., and Kazanietz, M. G. (2000) J. Biol. Chem. 275, 7574–7582
34. Emoto, Y., Kikami, H., Manome, Y., Kharbanda, S., and Kufe, D. (1996) Blood 87, 1990–1996
35. Emoto, Y., Manome, Y., Meinhartd, G., Kikami, H., Kharbanda, S., Robertson, M., Ghayur, T., Weng, W. W., Kamen, R., and Weichselbaum, R. (1995) EMBO J. 14, 6148–6156
36. Reyland, M. E., Anderson, S. M., Matassa, A. A., Barzen, K. A., and Quissell, D. O. (1999) J. Biol. Chem. 274, 19115–19123
37. Gubina, E., Rinaudo, M. S., Szallasi, Z., Blumberg, P. M., and Mufson, R. A. (1998) Blood 91, 823–829
38. Ohmori, S., Shirai, Y., Sakai, N., Fujii, M., Konishi, H., Kikkawa, U., and Saito, N. (1998) Mol. Cell. Biol. 18, 5263–5271
39. Isenbergs, J. S., and Klaunig, J. E. (2000) Toxicol. Sci. 53, 340–351
40. Macho, A., Decaudin, D., Castedo, M., Hirsch, T., Susin, S. A., Zamzami, N., and Kroemer, G. (1996) Cytometry 25, 333–340
41. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Cell 86, 147–157
42. Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D. R., Aebihl, R., Siderovski, D. P., Penninger, J. M., and Kroemer, G. (1999) Nature 397, 441–446
43. Engedal, N., Korkmaz, C. G., and Saatcioglu, F. (2002) Oncogene 21, 1017–1027
44. Strasser, A., O’Connor, L., and Dixit, V. M. (2000) Annu. Rev. Biochem. 69, 217–245
45. Scheel-Toellner, D., Pilling, D., Akbar, A. N., Hardie, D., Lombardi, G., Salmon, M., and Lord, J. M. (1999) Eur. J. Immunol. 29, 2603–2612
46. Soltoff, S. P. (2001) J. Biol. Chem. 276, 37986–37992
47. Hong, F., Nguyen, V. A., Shen, X., Kunos, G., and Gao, B. (2000) Biochem. Biophys. Res. Commun. 279, 974–979