An Endopolygalacturonase from *Sclerotinia sclerotiorum* Induces Calcium-Mediated Signaling and Programmed Cell Death in Soybean Cells

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A basic endopolygalacturonase (PG) isoform, produced early by *Sclerotinia sclerotiorum* when infecting soybean seedlings, was used to examine the signaling role of the enzyme in aequorin-expressing soybean cells. A cytosolic Ca^{2+} elevation was induced, with a rapid increase (phase 1) and a very slow decrease (phase 2) of Ca^{2+} concentration, indicating the involvement of Ca^{2+} ions in PG signaling. Within 1 h of PG-cell contact a remarkable level of cell death was recorded, significantly higher than the control cell culture turnover. The observed morphological and biochemical changes were indicative of the activation of programmed cell death; in particular, cytochrome *c* release in the cytoplasm and activation of both caspase 9-like and caspase 3-like proteases were found. When a polygalacturonase-inhibiting protein (PGIP) and the PG were simultaneously applied to cells, both the Ca^{2+} increase and cell death were annulled. The possible roles of prolonged sustained cytosolic Ca^{2+} concentrations in inducing cell death and of the PG-PGIP interaction in preventing PG signaling are discussed.

Additional keywords: fungal hydrolytic enzyme.

In plant-microbe interactions, the plant cell wall plays several roles, as a physical barrier to the pathogen infection, as a source of nutrients for the invaders, and as origin of signaling molecules. To overcome the plant cell wall, early during pathogenesis, most fungal pathogens secrete a wide array of cell wall-degrading enzymes with different substrate specificity, which facilitate the penetration of the pathogens into the host. The activity of pectin-degrading enzymes, in particular endopolygalacturonases (PGs) and endopectate lyases, is crucial for the colonization of plant tissues (Prade et al. 1999), and these enzymes can be determinants of pathogenicity or virulence (Willats et al. 2001). PGs hydrolyze internal $\alpha(1-4)$ glycosidic bonds in deesterified regions of the middle lamella and primary cell wall homogalacturonans and, when produced in relevant amounts, as by some necrotrophic fungi, determine

the maceration of infected tissues. PGs are often encoded by multigene families and produced in several molecular forms with different specificity and regulation. This multiplicity can confer adaptative flexibility to pathogens when infecting different hosts or plant organs (De Lorenzo et al. 2001).

Biologically active mixtures of oligogalacturonides (OGs) with various degrees of polymerization (from 2 to about 20 galacturonic acid residues) are released from the cell wall pectins by PGs (Ridley et al. 2001). OGs participate as endogenous signal molecules in the dialogue between host and microbe, activating in plants a cascade of physiological events that are at least partially Ca^{2+} -mediated (Navazio et al. 2002) and elicit defense responses (Coté and Hahn 1994).

Plants synthesize a class of proteins that specifically counteract the fungal PG activity. These polygalacturonase-inhibiting proteins (PGIPs) are located in the plant apoplast, in which, by complexing PGs, they reduce the homogalacturonan breakdown rate and the depolymerization of active OGs into shorter, inactive fragments (De Lorenzo and Ferrari 2002).

Sclerotinia sclerotiorum is a necrotrophic fungus causing soft rots in a number of host plants, and PG and oxalic acid, abundantly secreted by *S. sclerotiorum* in the host tissue, contribute to disease symptoms (Godoy et al. 1990; Marciano et al. 1983).

In this paper, we demonstrate that a basic PG isoform expressed early by *S. sclerotiorum* during soybean infection (Favaron and Marciano 1992; Favaron et al. 2004) induces, in aequorin-expressing soybean cells, a rapid and sustained cytosolic Ca^{2+} elevation that, in turn, leads to cell death exhibiting several hallmarks of programmed cell death (PCD). Both the Ca^{2+} increase and cell death induced by the PG are prevented by the simultaneous administration to cells of the PG-inhibitor PGIP, suggesting that PG and PGIP protein interaction alters the sensing of the fungal signaling molecule by the plant cells.

RESULTS

PG induces a sustained increase of intracellular Ca^{2+} concentration in soybean cells.

The PG purified from *S. sclerotiorum* was tested for its potential ability to elicit a Ca^{2+} response in soybean suspension cell cultures stably expressing aequorin. At a 0.5 enzyme reducing unit (RU) per milliliter dose, after a delay of 40 s, a rapid increase in cytosolic free- Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) was observed, reaching a peak of about 1.2 μM Ca^{2+} after

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about 2 min (phase 1) and slowly decreasing to a concentration of 0.6 μM within 10 min (phase 2) (Fig. 1). $[\text{Ca}^{2+}]_{\text{cyt}}$ remains at this value without declining to the basal level (approximately 100 nM) during the next hour (data not shown). The kinetic parameters of both phases of the Ca^{2+} transient were found to depend on the amount of PG applied (ranging from 0.05 to 1.0 RU/ml) (Fig. 1).

PG triggers cell death in soybean cells.

The viability of soybean cells treated with PG (0.5 RU/ml) was tested using the Evans blue method (Levine et al. 1996). The PG treatment was found to induce a twofold increase in the percentage of dead cells after 1 h of treatment ($22.8 \pm 0.4\%$) in comparison with untreated cells ($10.2 \pm 2.1\%$) (Fig. 2). Monitoring cell death over 12 h of PG treatment, we detected a progressive increase in the number of dead cells that reached nearly 100% after 12 h of PG incubation (Fig. 2).

Loss of cell viability was accompanied by changes in nuclear morphology, as examined by double staining of DNA with Hoechst 33342 (HO) and propidium iodide (PI) fluorescent dyes. In vivo, HO is a cell membrane-permeant dye that has access into slightly damaged as well as altered apoptotic

Fig. 1. Effect of endopolygalacturonase (PG) purified from *Sclerotinia sclerotiorum* on cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in soybean cells. At the time indicated (arrow) soybean cells stably expressing cytosolic aequorin were challenged with different doses of PG and the changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ were continuously recorded. These and the following traces are representative of three independent experiments, which gave very similar results.

Fig. 2. Viability of soybean cells treated with endopolygalacturonase (PG). Exponentially growing cells were incubated with PG (0.5 RU/ml) for different times (gray bars). Cells cultured without PG were taken as a control (white bar). The 100% value corresponds to cells treated for 10 min at 100°C . Data are means $\pm$ standard deviation of three independent experiments. Statistically significant at $P < 0.005$ (three asterisks).

nuclei, whereas PI is a cell-impermeant dye that can enter only in late apoptotic and necrotic cultured cells. These fluorochromes allow the discrimination of healthy (HO^-/PI^-), apoptotic (HO^+/PI^-), and necrotic (HO^+/PI^+) cells in an asynchronized cell population (Hamatake et al. 2000). After 1 h of PG treatment, about 15% HO^+/PI^- nuclei (Fig. 3E and F) and 20 to 30% HO^+/PI^+ nuclei (Fig. 3H and I) were observed, indicating chromatin condensation (HO positivity) and suggesting the simultaneous occurrence of both early and late apoptotic-like stages. In comparison, nuclei of untreated cells showed mainly the absence of HO and PI fluorescence, only about 5% of the cell population displayed positivity to both dyes (Fig. 3B and C).

Morphological observations under light microscopy revealed, in PG-treated cells, a gradual shrinkage of the nucleus and detachment of the plasma membrane from the cell wall as the cell death program proceeds (Fig. 3D and G). The detachment of the plasma membrane from the cell wall is considered one of the hallmarks indicating the occurrence of a PCD process in plant cells (Beers and McDowell 2001). Nuclei of control cells appeared larger and rounder compared with PG-treated cells, and the plasma membrane was completely adherent to the cell wall (Fig. 3A).

In order to investigate the mode of PG-induced cell death, all further analyses were carried out after 1 h of treatment. At this timepoint, in fact, the cell population is in a dynamic state toward PCD.

PG-induced cell death is associated with cytochrome *c* release and caspase-like protease activation.

To clarify the mechanism of cell death induced by PG, attention was focused on the cytochrome *c*/caspase 9 PCD pathway and downstream targets such as caspase 3. Cytochrome *c* release from mitochondria is an early event of apoptosis in animal cells. It is required for the activation of the caspase protease cascade as well as downstream events (Desagher and Martinou 2000). Cytosolic extracts from soybean cells were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were analyzed by Western blot with an anti-cytochrome *c* monoclonal antibody. In cytosolic

Fig. 3. Nucleus staining of endopolygalacturonase (PG)-treated cells. Untreated cells and cells treated for 1 h with PG (0.5 RU/ml) were stained with Hoechst (HO) and propidium iodide (PI) and were processed for fluorescence microscopy. A through C, untreated cells; D through I, cells treated with 0.5 RU/ml PG; E and F, early programmed cell death (PCD) nucleus; H and I, late PCD nucleus. Pictures represent typical examples. cc = chromatin condensation, cw = cell wall, nu = nucleus, and pm = plasma membrane. The arrow indicates the detachment of the plasma membrane from the cell wall.

extracts from cells treated with PG (0.5 RU/ml) for 1 h, the presence of cytochrome *c* was detected; control cells did not show any cytochrome *c* protein band (Fig. 4A). This result suggests the likely activation of a mitochondrial PCD pathway during PG treatment. To directly investigate whether PG-induced cell death is associated with the activation of caspase-like proteases, hydrolysis of the specific caspase 9 synthetic substrate LEHD and of the caspase 3 substrate DEVD, both conjugated with *p*-nitroaniline (pNA), was assayed in protein

Fig. 4. Cytochrome *c* release and measurements of caspase-like activities in soybean cells incubated with endopolygalacturonase (PG) (0.5 RU/ml) for 1 h. **A**, Cytochrome *c* accumulation in the cytosolic extracts from untreated (control) and PG-treated cells was assessed by Western blot analysis. **B**, Caspase 3-like activity in untreated cells (white bar), PG-treated cells (gray bar), and PG-treated cells in the presence of the caspase 3 inhibitor Ac-DEVD-CHO (3 μ M) (striped bar). **C**, Caspase 9-like activity in untreated cells (white bar), PG-treated cells (gray bar), and PG-treated cells in the presence of the caspase 9 inhibitor Ac-LEHD-CHO (3 μ M) (striped bar). Data are means $\pm$ standard deviation of three independent experiments. Statistically significant at $P < 0.05$ (one asterisk).

extracts from soybean cells. Caspase 9-like activity in cells treated with 0.5 RU/ml PG for 1 h was 1.86 ± 0.12 times greater than that in the control (Fig. 4B). Furthermore, incubation of cells with PG also heightened caspase 3-like activity by about 1.3 ± 0.03 times as compared with untreated cells (Fig. 4C). Although both analyzed caspase substrates were cleaved upon PG treatment, there was a quantitative difference between the two enzymatic reactions. The activity of caspase 9-like protease seemed to be about 1.4-fold higher than that of caspase 3-like protease (Fig. 4B and C). Both activities of caspase 9-like and caspase 3-like proteases were significantly decreased by the Ac-LEHD-CHO and Ac-DEVD-CHO inhibitors of animal caspase 9 and 3, respectively (Fig. 4B and C). Thus, the mechanism of cell death induced by PG seems to involve cytochrome *c* release, caspase 9-like and caspase 3-like activation, suggesting the occurrence of a PCD process.

PGIP blocks both the PG-induced Ca^{2+} signaling and PCD.

PGIP is constitutively present in the tissues of soybean seedlings (Favaron et al. 1994), and it is secreted in the culture medium by soybean cells (data not shown). However, the washing procedure carried out before the PG treatment freed the cell of any detectable PGIP activity. Therefore, exogenous PGIP (2 inhibitor units [IU] per milliliter) was administered to cell suspensions simultaneously with the PG treatment (0.5 RU/ml).

The simultaneous administration to cells of PG and its inhibitor PGIP resulted in the almost complete annulment of the PG-induced Ca^{2+} transient, with a remarkable reduction of phase 1 amplitude and the complete abolishment of the phase 2 plateau (Fig. 5A). It has to be noted that PGIP by itself could not elicit any significant $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation over the basal level. Indeed,

Fig. 5. Polygalacturonase inhibiting protein (PGIP) effectively blocks endopolygalacturonase (PG)-induced Ca^{2+} signaling. **A**, Cells were treated (arrow) with either PG (0.5 RU/ml) alone (black trace) or PG (0.5 RU/ml) and PGIP (2 inhibitor units per milliliter) administered simultaneously (gray trace) and their effect on cytosolic free- Ca^{2+} concentration $[\text{Ca}^{2+}]_{\text{cyt}}$ was monitored. **B**, Cells were challenged (arrow) with PGIP (black trace) or culture medium containing the same percentage of the solvent as PGIP-treated cells (gray trace).

the PGIP-induced Ca^{2+} trace appeared almost superimposable to that of control cells challenged with culture medium (Fig. 5B).

Figure 6 shows that PGIP effectively blocks the activation of the PG-induced PCD program, as demonstrated by the Evans blue cell viability assay (Fig. 6A) and double staining with HO and PI (Fig. 6B, panels e and f). Cell treatment with only PGIP did not induce either a decrease of cell viability (Fig. 6A) or positivity to HO and PI (Fig. 6B, panels b and c).

The cytosolic Ca^{2+} elevation is a key determinant of the PG-induced cell death.

To investigate the role of Ca^{2+} in PG-activated signaling, the Ca^{2+} channel blocker La^{3+} , which has been previously demonstrated to effectively abolish the Ca^{2+} transient elicited by OGs (Navazio et al. 2002), was applied to soybean cells 10 min before treatment with PG. The inhibitor markedly reduced both phase 1 and 2 of the Ca^{2+} transient (Fig. 7A). The annulment of the Ca^{2+} signal in La^{3+} -pretreated cells abolished both caspase 3-like activation (Fig. 7B) and cell death (Fig. 7C), which did not significantly differ from that of control cells. Moreover, the number of HO/PI positive cells was comparable to the control (Fig. 7D), highlighting the absence of chromatin condensation in PG-treated cells after incubation with La^{3+} .

Fig. 6. Polygalacturonase inhibiting protein (PGIP) effectively blocks endopolygalacturonase (PG)-induced cell death. **A**, Exponentially growing cells were incubated for 1 h with PG (0.5 RU/ml) (gray bar), PGIP (2 inhibitor units [IU] per milliliter) (striped bar), or a mixture of PG (0.5 RU/ml) plus PGIP (2 IU/ml) (black bar). Cells cultured without PG and PGIP were taken as a control (white bar). Data are means $\pm$ standard deviation of three independent experiments. Statistically significant at $P < 0.005$ (three asterisks). **B**, Cells treated for 1 h with PGIP (2 IU/ml) and with PG (0.5 RU/ml) plus PGIP (2 IU/ml) were stained with Hoechst (HO) and propidium iodide (PI) dyes. **a** through **c**, PGIP-treated cells; **d** through **f**, PG plus PGIP-treated cells. Pictures represent typical examples. nu = nucleus.

DISCUSSION

In this paper, we show that administering to soybean cell cultures a PG produced by *Sclerotinia sclerotiorum* when infecting soybean plants triggers a rapid elevation in $[\text{Ca}^{2+}]_{\text{cyt}}$, which is suggestive of Ca^{2+} -mediated signaling induced by a secreted fungal enzyme. This enzyme in vivo degrades the pectic components of the plant cell wall, generating oligosaccharide fragments, which in turn, act as endogenous signaling molecules (Coté and Hahn 1994). The mixture of OGs deriving from PG enzymatic activity is known to activate a Ca^{2+} -mediated signal pathway (Navazio et al. 2002). Nevertheless, the temporal kinetics of the OG-elicited Ca^{2+} transient significantly differs from that induced by treatment with PG. In particular, although both elicitors evoke a single immediate Ca^{2+} spike, the OG-activated Ca^{2+} transient is rapidly dissipated within a few minutes (Navazio et al. 2002), whereas the PG-activated Ca^{2+} signal persists for at least 1 h, maintaining, in the cell, Ca^{2+} levels higher than the physiological ones. In both animal and plant cells, perturbations of Ca^{2+} homeostasis have been associated with cytotoxic events (Kass and Orrenius 1999; White and Broadley 2003).

Here, we provide evidence for the occurrence of PCD induced by PG in soybean cells. It must be noted that the products of PG enzymatic activity, namely OGs, do not by themselves induce cell death in either soybean cells (data not shown) or other experimental systems (Binet et al. 2001; Lecourieux et al. 2002; Shibuya and Minami 2001). Thus, PG seems to be, per se, the causative agent of the cell death, which could depend on the cell sensing of the protein as a virulence factor and may be unrelated to its enzymatic activity. Likewise, the elicitor activity of other fungal hydrolytic enzymes has been previously shown by various experimental approaches not to be necessarily due to the release of biologically active oligosaccharide fragments (Carden and Felle 2003; Martinez et al. 2001; Poinsett et al. 2003; Rouet-Mayer et al. 1997; Yano et al. 1998).

The elevation of cytosolic Ca^{2+} level seems to be essential for the induction of cell death. Indeed, the Ca^{2+} channel blocker La^{3+} abolishes both phases of the PG-induced Ca^{2+} transient, caspase 3-like activation, chromatin condensation, and cell death. A relationship between sustained $[\text{Ca}^{2+}]_{\text{cyt}}$ and activation of pathogen defense responses has been previously reported (Grant et al. 2000).

The sequence of events starting from signal perception to cell death in PG-treated cells has been partially unraveled in this paper. Based on previous findings by several authors (Boudart et al. 2003; Cotton et al. 2002; Poinsett et al. 2003), it can be argued that, downstream of the Ca^{2+} transient, an extracellular H_2O_2 accumulation can occur. Both $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation (Viroleinen et al. 2002) and production of reactive oxygen species (Tiwari et al. 2002) may be cocausative agents in triggering PCD through the mediation of mitochondria. Our data provide evidence for the release, in the cytoplasm, of cytochrome *c* as well as activation of caspase-like proteases in PG-induced cell death. Nevertheless, the involvement of other proteases, such as the newly characterized serin proteases named saspases (Coffeen and Wolpert 2004), cannot be ruled out.

The complete block of both PG-induced Ca^{2+} transient and PCD following the interaction of PG with its inhibitor PGIP is consistent with an interference of PGIP with signaling by PG. PGIP, by complexing PG, besides blocking the substrate admittance to the enzyme-active site (Di Matteo et al. 2003), might prevent the ligand access to the putative receptor, hampering its recognition. Consequently, any cellular receptor-mediated response to the elicitor is annulled. The inhibition of H_2O_2 production shown by Poinsett and associates (2003), consequent

to the simultaneous administration of PG and PGIP to grapevine cells, gives support to this hypothesis. An open question remains whether the PGIP role in the modulation of PG functioning is really dual (i.e., block of PG recognition and of its enzymatic activity) or if, rather, all PG-induced cell responses are prevented by the failed PG interaction with its receptor. Poinsett and associates (2003) speculated that the PG catalytic domain and the recognition motif might correspond. Others have also suggested (De Lorenzo et al. 1997; Esquerré-Tugayé 2000; James and Dubery 2001) that PGIP might act as a cell-surface receptor or, at least, be part of a receptor complex.

In conclusion, a PG produced early during plant infection by *S. sclerotiorum* is a candidate factor for triggering pathogen-induced PCD. As recently reported, this necrotrophic fungus seems to co-opt PCD response in host cells to produce disease symptoms (Dickman et al. 2001; Govrin and Levine 2000), and the expression of animal antiapoptotic genes in transgenic tobacco plants prevents fungal colonization and disease development (Dickman et al. 2001).

The plant PGIP blocking the PCD induced by this *S. sclerotiorum* PG may be a successful instrument to improve plant resistance to this pathogen.

MATERIALS AND METHODS

Plant material.

Soybean (*Glycine max* L.) suspension cell cultures stably expressing cytosolic aequorin (Mithöfer et al. 1999) were provided by G. Neuhaus (Freiburg, Germany). Cells were cultivated in Murashige and Skoog liquid medium supplemented with 0.5% sucrose, 10 µg naphthaleneacetic acid per milliliter, 2 µg of kinetin per milliliter, 1 µg of thiamine-HCl per milliliter, and 10 µg of kanamycin per milliliter on a rotary shaker (80 rpm) at 24°C under a 16-h photoperiod and were subcultured every three weeks with a 10% (vol/vol) inoculum.

Fungus and plant infection.

The B-24 isolate of *S. sclerotiorum* (Lib. De Bary) was grown on potato dextrose agar medium to obtain mycelium for soybean inoculation. Six-day-old etiolated soybean plants were obtained at 25°C in sterilized moist vermiculite. Soybean seedlings were inoculated by placing plugs (10 × 2 mm) of mycelium-colonized agar, cut from marginal zones of colonies, along the middle region of hypocotyls. The trays

Fig. 7. La^{3+} inhibition of endopolygalacturonase (PG)-triggered cytosolic Ca^{2+} concentration $[Ca^{2+}]_{cyt}$ elevations, caspase 3-like activity, chromatin condensation, and cell death. **A**, Cells were incubated with 3 mM La^{3+} (gray trace) for 10 min prior to the addition of PG (0.5 RU/ml) (arrow), and the Ca^{2+} response was monitored. Control cells were treated only with PG (black trace). **B**, Caspase 3-like activity and **C**, viability of soybean cells treated for 1 h with PG (0.5 RU/ml) alone (black bar) or 3 mM La^{3+} (striped bar) or La^{3+} plus PG (gray bar). Cells cultured without PG and La^{3+} were taken as a control (white bar). Data are means $\pm$ standard deviation of three independent experiments. Statistically significant at $P < 0.05$ (one asterisk) and $P < 0.005$ (three asterisks). **D**, Cells treated for 1 h with PG (0.5 RU/ml) after a 10-min pretreatment with 3mM La^{3+} were stained with Hoechst and propidium iodide dyes. **a** through **c**, Control cells; **d** through **f**, cells treated with La^{3+} ; **g** through **i**, cells treated with La^{3+} plus PG. Pictures represent typical examples. Cells cultured without PG and La^{3+} were taken as a control.

containing inoculated seedlings were sealed with plastic film and were incubated at 25°C in the dark. Infected tissue was harvested for PG extraction and purification 48 h after inoculation.

PG extraction, purification and assay.

A basic PG isoform of *S. sclerotiorum* was extracted and purified from infected soybean tissue as reported (Favaron et al. 1997). Briefly, the PG was purified by two successive preparative isoelectrofocusing (IEF) runs in the range of pH 3 to 10 and 6 to 9, respectively, and by a MonoS cation-exchange column. PG activity was determined as an increase in reducing end groups over time. Reducing end groups were measured using the method described by Milner and Avigad (1967), with D-galacturonic acid as a standard. The incubation mixture contained 200 µl of 0.5% polygalacturonic acid (PGA, from orange, code P3889, lot 85C-0121; Sigma-Aldrich, St. Louis) in 100 mM sodium acetate buffered at pH 4.6 and 0.1 mg of bovine serum albumin per milliliter. One RU was defined as that producing 1 µmol of reducing end groups per minute at 30°C and corresponds to about 1.4 µg of PG protein.

PGIP production and assay.

PGIP was obtained from leaves of 14-day-old plants of *Phaseolus vulgaris* L. (cv. Borlotto). Proteins were extracted and PGIP was purified by preparative IEF (pH 3 to 10), PG-Sepharose affinity chromatography, and MonoS cation-exchange chromatography as previously reported (Favaron et al. 1994). PGIP activity was determined against the purified PG of *S. sclerotiorum*, by adding the inhibitor to the enzyme mixture described in the PG-reducing end groups assay. One IU was defined as that required to reduce by 50% the activity of one PG unit. About 4 IU (approximately 3.2 µg of PGIP) were used in order to abolish completely 1 PG unit.

PG treatment.

Cell treatments with the *S. sclerotiorum* PG were performed three weeks after reinoculation, during the exponential growth phase of the cells. Cells were washed three times with 10 vol of fresh hormone-free culture medium and were used after 30 min of equilibration. Control cells were incubated in the same conditions without the elicitor.

Aequorin-dependent Ca²⁺ measurements.

Reconstitution of the Ca²⁺-sensitive photoprotein aequorin was carried out by incubating soybean cells with 5 µM coelenterazine overnight, in the dark. All measurements were performed at room temperature in a final volume of 50 µl, containing approximately 5 mg (fresh weight) of reconstituted cell-suspension culture. Treatment with PG was carried out by injecting, with a light-tight syringe through the luminometer port, an equal volume of twofold-concentrated stock solutions (dissolved in the basal cell culture medium) into the cell suspension culture. All experiments were terminated by discharging the remaining aequorin pool with 0.33 M CaCl₂ in 10% ethanol. Luminescence data were collected and elaborated into [Ca²⁺] values by a computer algorithm based on the Ca²⁺ response curve of aequorin (Brini et al. 1995).

Cell viability and treatments.

Cell viability was determined by incubation with 0.05% Evans blue (Levine et al. 1996). Cells were then extensively washed with deionized water to remove excess and unbound dye. Dye bound to dead cells was solubilized in methanol/SDS (50:1) for 30 min at 50°C and was quantified by absorbance at 600 nm.

Nucleus staining.

To analyze nuclear morphological changes, cells were stained with fluorescence dyes HO and PI. Suspension cells (3-week-old) were incubated in darkness with 8 µg of HO per milliliter (Sigma-Aldrich) and 5 µg of PI per milliliter (Sigma-Aldrich) at room temperature for 10 min and were observed under fluorescence microscopy, by using an excitation light of 350 nm and 570 nm, respectively, for the two dyes.

Detection of cytochrome c release.

Cytosolic protein extracts were obtained by incubating cells (15 min at 4°C) with 75 mM NaCl, 1 mM NaH₂PO₄, 8 mM Na₂HPO₄, 250 mM sucrose, and digitonin (380 µg/ml). Cytosolic fractions were then separated by centrifugation (5 min, 14,000 rpm, 4°C), were loaded (10 µg) on SDS-PAGE (12% acrylamide gel) (Laemmli 1970), and were transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA, U.S.A.) for chemiluminescent detection according to Towbin and associates (1979). Filters were incubated with 5% skim milk in Tris-buffered saline (TBS) for 1 h and were probed with a mouse monoclonal cytochrome c antibody (4 µg/ml) (BioSource International, Camarillo, CA, U.S.A.) for 2 h. After incubation, filters were washed two times with a 0.05% Tween/TBS solution, followed by one wash in TBS before incubation with the secondary antibody. Labeled proteins were detected using CDP-star (Biolabs, Hitchin, U.K.) and autoradiography films (Sigma-Aldrich).

Measurements of caspase-like activities.

Caspase 9- and caspase 3-like activities were measured using the Caspase-9 and Caspase-3 colorimetric activity assay kits (Chemicon International, Inc., Temecula, CA, U.S.A.), respectively, as described by the manufacturer. The assays are based on spectrophotometric detection of the chromophore pNA after the caspase-dependent cleavage from the labeled substrates DEVD-pNA or LEHD-pNA by active caspase 3 or 9, respectively. Cytosolic protein extracts from soybean cells (15 µg) were incubated for 2 h at 37°C with DEVD-pNA or LEHD-pNA, in the presence or absence of 3 µM caspase inhibitors Ac-DEVD-CHO (for caspase 3) or Ac-LEHD-CHO (for caspase 9). Caspase-like activity was determined by spectrophotometric quantification of the free pNA (λ_{em} = 405 nm).

Statistical analysis.

Data are presented as mean plus or minus standard deviation of three independent experiments, and the differences between groups were assessed by Student's *t*-test.

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