JOURNAL OF CELLULAR PHYSIOLOGY 209:142–152 (2006)

Early Caspase-3 Activation Independent of Apoptosis Is Required for Cellular Function

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A number of pro-apoptotic stimuli induce the activation of caspase-9, an initiator protease that activates executioner caspases, such as caspase-3, leading to the development of programmed cell death. Here we demonstrate that cell (platelets and pancreatic acinar cells) stimulation with agonists induces a bimodal activation of caspase-3. The early caspase-3 activation occurs within 1 min of stimulation and is independent on caspase-9 or mitochondrial cytochrome c release suggesting that is a non-apoptotic event. The ability of agonists to induce early activation of caspase-3 is similar to that observed for other physiological processes. Activation of caspase-3 by physiological concentrations of cellular agonists, including thrombin or CCK-8, is independent of rises in cytosolic calcium concentration but requires PKC activation, and is necessary for agonist-induced activation of the tyrosine kinases Btk and pp60^{src} and for several cellular functions, including store-operated calcium entry, platelet aggregation, or pancreatic secretion. Thus, early activation of caspase-3 seems to be a non-apoptotic event required for cellular function. J. Cell. Physiol. 209: 142–152, 2006.

One of the earliest and most consistently observed features of apoptosis is the activation of a series of cytosolic cysteine proteases, called caspases, which cleave multiple protein substrates *en mase*, leading to the loss of cellular structure and function, and ultimately resulting in cell death (Stennicke and Salvesen, 1998). The caspase family consists of at least 14 enzymes in mammalian cells. In particular, caspases-3, -8, and -9 play a relevant role in apoptosis: caspase-9 in the mitochondrial pathway, caspase-8 in the Fas/CD95 pathway, and caspase-3, more downstream, is an executioner caspase-activated by multiple pathways (Shi, 2002).

Traditionally, two general pathways to apoptosis have been described. One pathway is the so-called extrinsic pathway initiated by the binding of an extracellular death ligand, such as FasL, to its cell-surface death receptor, such as Fas (Ashkenazi and Dixit, 1998). The second pathway is the intrinsic pathway, which is mediated by mitochondria. In response to apoptotic stimuli, several proteins are released from the intermembrane space of mitochondria into the cytoplasm (Green and Reed, 1998). Some of well-characterized proteins include cytochrome c, which mediates the activation of caspase-9 (Li et al., 1997), triggering a cascade of caspase-activation, including caspase-3, which promotes cellular apoptosis. However, some studies have reported apoptosis-independent pathway of caspase-activation. Caspase-3 activity has been found in T lymphocytes stimulated by mitogens without evidence that apoptosis is proceeding (Wilhelm et al., 1998), and caspase-3-like protease functions have been found in terminal differentiation of lens epithelial cells (Ishizaki et al., 1998), which represent an additional example of caspase function in a non-apoptotic setting.

In the present study we have investigated the effect of physiological agonists on rapid activation of caspase-3 independently of the development of apoptosis, as detected by phosphatidylserine (PS) exposure (Shcherbina and Remold-O'Donnel, 1999; Wolf et al., 1999). We demonstrate that agonists, such as thrombin or CCK-8, are able to induce rapid activation of caspase-3, which is required for full stimulation of cellular function. Furthermore, we demonstrate that caspase-3 activity is required for store-operated Ca^{2+} entry (SOCE) in platelets, an event that might be mediated by the activation of Bruton's tyrosine kinase (Btk) and pp60^{src}.

MATERIALS AND METHODS

Materials

Fura-2 acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Leiden, The Netherlands). Apyrase (grade VII), aspirin, BME vitamin mixture, BME amino acid mixture, thrombin, CCK-8, TG, acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (AC-DEVD-AMC), Z-Ile-Glu(OMe)-Thr-DL-Asp(OMe)-fluoromethylketone (Z-IETD-FMK), Z-Ala-Glu-Val-DL-Asp-fluoromethylketone (Z-AEVD-FMK), dithiothreitol (DTT), trypsin inhibitor, *N*-acetyl-Leu-Glu-His-Asp-7-amido-4-trifluoromethylcoumarin (AC-LEHD-AFC), CHAPS, bongkrekic acid, dimethyl BAPTA, fluorescein isothiocyanate-labeled annexin V, glutaraldehyde, and BSA were from Sigma (Poole, Dorset, UK). Iono and Ro-31-8220 were from Calbiochem (Nottingham, UK). Collagenase CLSPA was obtained from Worthington Biochemicals (Lakewood, NJ). DEVD-CMK and z-LEHD-FMK were from BD biosciences (Madrid, Spain). Phadebas Reagent test was from Amersham (Arlington Heights, IL). Anti-phospho-Btk (Y-223) antibody was from Cell Signaling Technology (Beverly, MA). Anti-phospho-c-Src (Y-416) antibody, anti-Btk antibody (N-20), and horseradish peroxidase-conjugated goat anti-rabbit and donkey anti-goat IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pp60^{src} (GD11) antibody was from Upstate (Barcelona, Spain). All other reagents were of analytical grade.

Contract grant sponsor: MCyT-DGI; Contract grant number: BFU2004-00165; Contract grant sponsor: DGI fellowship; Contract grant number: BFU2004-00165.

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Received 4 May 2006; Accepted 25 May 2006

DOI: 10.1002/jcp.20715

Platelet preparation

Platelet suspensions were prepared as described previously (Rosado et al., 2000b) as approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Briefly, blood was obtained from healthy drug-free volunteers and mixed with one-sixth volume of acid/citrate dextrose anticoagulant containing (in mM): 85 sodium citrate, 78 citric acid, and 111 D-glucose. Platelet-rich plasma was then prepared by centrifugation for 5 min at 700*g* and aspirin (100 μM) and apyrase (40 μg/ml) were added. For loading with dimethyl BAPTA, cells were incubated for 30 min at 37°C with 10 μM dimethyl BAPTA AM. Cells were then collected by centrifugation at 350*g* for 20 min and resuspended in HEPES-buffered saline (HBS), pH 7.45, containing (in mM): 145 NaCl, 10 HEPES, 10 D-glucose, 5 KCl, 1 $MgSO_4$, and supplemented with 0.1% BSA and 40 μg/ml apyrase.

Preparation of isolated pancreatic acinar cells

Adult male Swiss mice were obtained from the Animal Farm, Faculty of Veterinary Sciences, UEX, Spain. Mice were sacrificed by cervical dislocation, the pancreas was rapidly removed and the acinar cells were isolated as described previously (Redondo et al., 2003). Briefly, the pancreas was incubated in the presence of collagenase for 5–10 min at 37°C under gentle agitation. The enzymatic digestion of the tissue was followed by gently pipetting the cell suspension for mechanical dissociation of the acinar cells. After centrifugation, cells were resuspended in HBS supplemented with 1 mM $CaCl_2$, 1% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture, and 1% (v/v) amino acid mixture. In experiments performed in Ca^{2+}-free medium cells were resuspended in HBS containing 100 μM $CaCl_2$ and 200 μM EGTA was added at the time of experiment. All experimental procedures were approved by the local ethical committee.

Caspase activity assay

To determinate caspase-3 and -9 activities, stimulated or resting cells were sonicated, and cell lysates were incubated with 2 ml of substrate solution (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, and 8.25 μM of caspase substrate) for 2 h at 37°C as described previously (Ben Amor et al., 2006). Substrate cleaving was measured with a fluorescence spectrophotometer with excitation wavelength of 360 nm and emission at 460 nm for caspase-3 and excitation wavelength of 400 nm and emission at 505 nm for caspase-9. Preliminary experiments reported that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors or caspase-3 or -9, respectively. The activity of caspases was calculated from the cleavage of the respective specific fluorogenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AFC for caspase-9) following the instruction of the manufacturer.

Determination of phosphatidylserine externalization

The PS exposure of resting and stimulated platelets and pancreatic acinar cells was determined according to a previously published procedure (Shcherbina and Remold-O'Donnel, 1999; Wolf et al., 1999). Briefly, cells were stimulated in HBS. Samples of cell suspensions (500 μl) were transferred to 500 μl ice-cold 1% (w/v) glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. Cells were then incubated for 10 min with annexin V-FITC (0.6 μg/ml) in PBS supplemented with 0.5% (w/v) bovine serum albumin. After incubation the cells were collected by centrifugation for 60 sec at 3,000*g* and resuspended in PBS. Cell staining was measured using a Shimadzu spectrofluorimeter. Samples were excited at 496 nm and emission was recorded at 516 nm.

Measurement of intracellular free calcium concentration ($[Ca^{2+}]_c$)

Human platelets were loaded with fura-2 by incubation with 2 μM fura-2/AM for 45 min at 37°C. Fluorescence was recorded from 2 ml aliquots of magnetically stirred cellular suspension (2×10^8 cells/ml) at 37°C using a Cary Eclipse Spectrophotometer (Varian Ltd, Madrid, Spain) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (1985).

Ca^{2+} entry was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after addition of $CaCl_2$ (Rosado et al., 2000b). Ca^{2+} release was estimated using the integral of the rise in $[Ca^{2+}]_c$ for 2.5 min after agonist addition. Both Ca^{2+} entry and release are expressed as nM · sec, as previously described (Heemskerk et al., 1997; Rosado and Sage, 2000a). Ca^{2+} entry was corrected by subtraction of the $[Ca^{2+}]_c$ elevation due to leakage of the indicator.

Western blotting

Cell stimulation was terminated by mixing with an equal volume of 2× Laemmli's buffer (Laemmli, 1970) with 10% DTT followed by heating for 5 min at 95°C. One-dimensional SDS-electrophoresis was performed with 10% SDS–PAGE and separated proteins were electrophoretically transferred, for 2 h at 0.8 mA/cm^2, in a semi-dry blotter (Hoefer Scientific, Newcastle, Staffs., UK) onto nitrocellulose for subsequent probing. Blots were incubated overnight with 10% (w/v) BSA in tris-buffered saline with 0.1% Tween 20 (TBST) to block residual protein binding sites. Blocked membranes were then incubated with the anti phospho-Btk (Y-223), the anti-Btk (N-20), or anti-pp60^{src} (GD11) antibodies diluted 1:1,000 in TBST for 2 h or with the anti-phospho-c-Src (Y-416) antibody diluted 1:600 in TBST for 1 h. The primary antibody was removed and blots washed six times for 5 min each with TBST. To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:10,000 in TBST, washed six times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films and the optical density was estimated using scanning densitometry. Membranes probed with the anti-phospho-Btk or the anti-phospho-c-Src antibodies were stripped of bound antibodies by incubating in stripping buffer (0.1 M glycine, pH 2.2, 1% Tween 20, 0.1% SDS) at room temperature with agitation and reprobed with the anti-Btk or anti-pp60^{src} antibodies, respectively.

Platelet aggregation

Platelets prepared as described above were suspended in HBS supplemented with 0.1% w/v bovine serum albumin. Platelet aggregation was monitored in a Chronolog (Havertown, PA) aggregometer at 37°C under stirring at 1,200 rpm (Redondo et al., 2005a).

Amylase release

Amylase release in pancreatic acinar cells was measured using the procedure published previously (Rosado et al., 1998). Amylase activity was, unless otherwise stated, determined after a 30 min incubation with CCK-8 using the Phadebas reagent. Amylase release was calculated as the percentage of the amylase activity in the acini at the beginning of the incubation that was released into the extracellular medium during the incubation.

Statistical Analysis

Analysis of statistical significance was performed using Student's *t*-test. $P < 0.05$ was considered to be significant for a difference.

RESULTS

Thrombin and store depletion by TG + Iono activate caspase-3 and -9 in human platelets

In our effort to investigate the role of caspases-3 and -9 in cellular biology, we analyzed caspase-activation upon cell stimulation. Caspases-3 and -9 have been reported to play a functional role in human platelets (Shcherbina and Remold-O'Donnel, 1999; Wolf et al., 1999). Treatment of human platelets, an anucleated cell model, with 1 U/ml thrombin induced a time-dependent bimodal activation of caspase-3, which consisted of an early

activation within 1 min of stimulation, with a 4.8 ± 0.6 (SE)-fold increase, then a decrease reaching a minimum at 10 min after stimulation, and then a delayed increase in caspase-3 activity reaching a maximum after 2 h of stimulation with a 7.2 ± 0.7-fold increase (Fig. 1A). Similar results were observed with a lower concentration of thrombin (0.01 U/ml; Fig. 1A). Activation of caspase-9 by thrombin showed a less marked bimodal pattern than that of caspase-3, reaching a delayed initial peak of caspase-9 activity after 10 min of stimulation, with a 2.6 ± 0.1-fold increase, then a decrease with a minimum at 30 min and then showed an increase that was maximal after 2 h with a 5.0 ± 0.3-fold increase (Fig. 1A). Thrombin is a physiological agonist that activate platelet function by hydrolysis of phosphatidylinositol 4,5-bisphospate (PIP_2) and generation of lipid messengers, synthesis of IP_3 and subsequent Ca^{2+} release from the intracellular stores (Rink and Sage, 1990). Extensive depletion of the intracellular Ca^{2+} stores, using thapsigargin (TG; 1 μM) in combination with a low concentration of ionomycin (Iono; 50 nM) as previously described (Cavallini et al., 1995; Rosado and Sage, 2000b), induced a time-dependent and bimodal caspase-3 activation similar to that observed after cell stimulation with thrombin, reaching an early activation within 1 min, with a 3.9 ± 0.4-fold increase and then a late activation that was maintained for at least 2 h (Fig. 1B). In contrast, activation of caspase-9 by TG + Iono was slower and maintained during the time of the experiment (2 h; Fig. 1B). Although caspase-3 has been shown to be a downstream effector of caspase-9-mediated apoptotic events, the early activation of caspase-3 was not found to correlate with a previous activation of caspase-9, which suggests that this event might be a physiological rather than apoptotic process.

This hypothesis received support from our studies exploring the concentration-dependent effect of thrombin on caspase-3 and -9 activation at early (1 min) and late (60 min) times. As shown in Figure 1C,D, the effect of thrombin on caspase activation was concentration dependent. Cell stimulation with thrombin for 1 min caused a detectable activation of caspase-3 at 0.01 U/ml

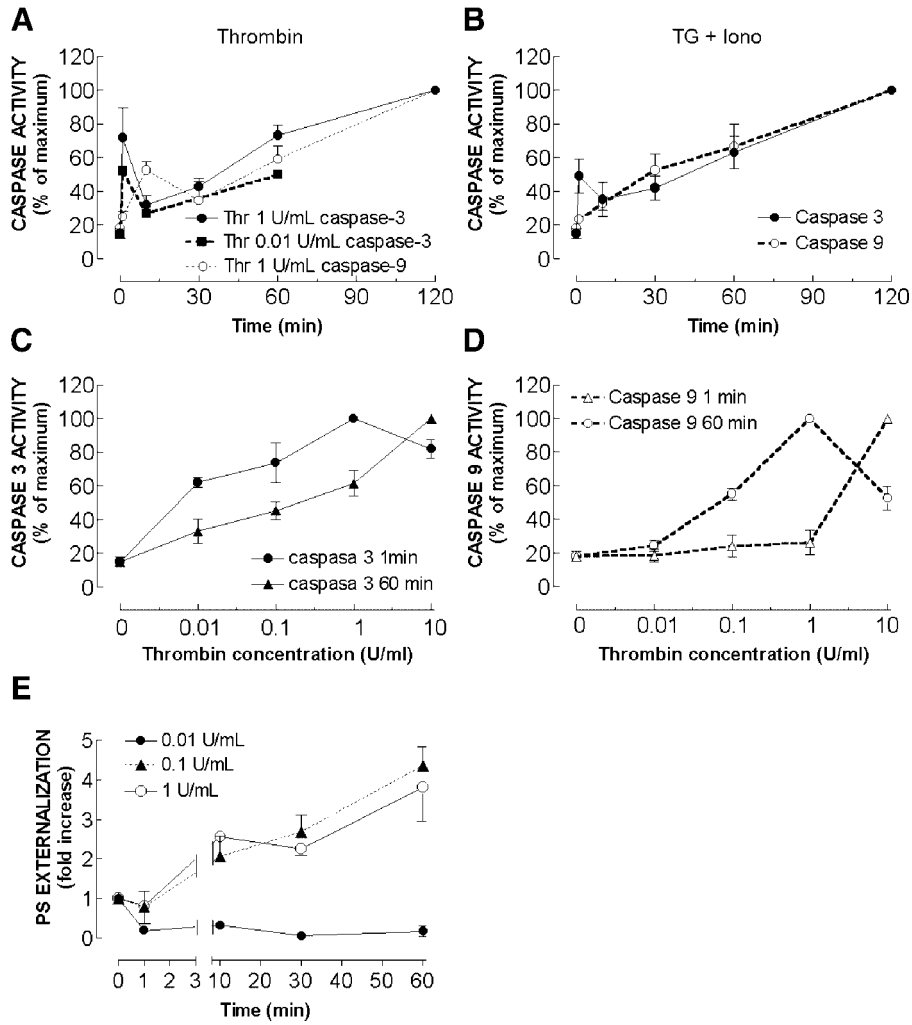

Fig. 1. Time course and concentration dependence of thrombin- and TG + Iono-induced activation of caspase-3 and caspase-9 and phosphatidylserine (PS) exposure in human platelets. Human platelets were stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) for various periods of time with thrombin (**A**) or with TG (1 μM) combined with Iono (50 nM; (**B**) or for either 1 or 60 min with increasing concentrations of thrombin (0.01–10 U/ml; **C** and **D**). Caspase-3 and -9 activities were analyzed following the caspase activity assay based on the cleavage of their respective specific fluorogenic substrates as described under Material and Methods. Values are presented as mean ± SE (n = 6) expressed as the percentage of maximal increase caused by thrombin or TG + Iono above Control unstimulated values. **E**: Platelets were stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with increasing concentrations of thrombin (0.01–1 U/ml) for various periods of time and PS exposure was determined as described in Material and Methods. Values are presented as mean ± SE (n = 6) expressed as fold-increase over the pretreatment level (experimental/control).

and a maximal effect at 1 U/ml, and then decreased by up to approximately 20% with supramaximal concentrations of thrombin (Fig. 1C). In contrast, higher concentrations of thrombin were required to induce maximal activation of caspase-3 after 60 min of stimulation, with a half-maximal effect at 1.93 U/ml (Fig. 1C). In contrast to the late caspase-3 activation, thrombin induced rapid activation of caspase-3 at concentrations similar to that found to activate physiological processes, such as Ca^{2+} mobilization (Heemskerk et al., 1997) or aggregation (Jakubowski and Maraganore, 1990), suggesting that this event might be a physiological process.

Caspase-9 is an initiator caspase involved in the initial steps of apoptosis (Li et al., 1997). Activation of caspase-9 by cell stimulation with thrombin for 1 min was undetectable at the concentration 0.01 U/ml, and was found to be maximal at 10 U/ml (Fig. 1D). Cell stimulation with thrombin for 60 min induced a biphasic effect on caspase-9 activity, showing a detectable caspase-9 activation at 0.01 U/ml, reaching a maximal effect at 1 U/ml and decreasing by up to approximately 50% by supramaximal concentrations of thrombin (Fig. 1D). These findings indicate that low concentrations of thrombin induce rapid activation of caspase-3 followed by a slower activation of caspase-9 that might be responsible for the late activation of caspase-3.

In order to investigate whether the early activation of caspase-3 by thrombin is an apoptotic event we have checked the apoptotic state of platelets by testing PS exposure. As shown in Figure 1E, treatment of platelets with thrombin for 1 min did not induce PS externalization, which further confirms that the early activation of caspase-3 is not associated with apoptosis. In addition thrombin, at 0.01 U/ml, was unable to induce apoptosis (Fig. 1E). Higher concentrations of thrombin (0.1 and 1 U/ml) induced a time-dependent activation of PS externalization, which, in the case of 1 U/ml thrombin, was parallel to the activation of caspase-9 (Fig. 1A,E; $n = 6$).

Thrombin-induced rapid activation of caspase-3 is independent of caspases-8, -9, and -10

We have further investigated whether the early activation of caspase-3 is dependent on caspase-9 activity by using two different means: inhibition of caspase-9, using the specific inhibitor z-LEHD-FMK (Ozoren et al., 2000), and blockade of cytochrome c release through the mitochondrial transition pore, using bongkrekic acid, which has been shown to prevent apoptosis-dependent activation of caspase-9 and -3 (Gerasimenko et al., 2002). Cell incubation for 90 min with 40 μM z-LEHD-FMK abolished thrombin-induced caspase-9 activation (data not shown; $n = 6$). Consistent with the results presented above, incubation with the caspase-9 inhibitor had no significant effects on thrombin-evoked early caspase-3 activation, while abolished the late activation of caspase-3 (Fig. 2A; $n = 6$). In agreement with this, treatment for 45 min with 50 μM bongkrekic acid did not impair early caspase-3 activation by thrombin but prevented late activation of caspase-3 (Fig. 2A; $n = 6$). These findings strongly suggest that thrombin induces early activation of caspase-3 independently of caspase-9 and mitochondrial-dependent apoptosis. In contrast,thrombin-evoked late caspase-3 activation is likely an apoptotic event dependent on the activation of caspase-9 by mitochondrial cytochrome c release.

We have further investigated whether early caspase-3 activation is dependent on other initiator caspases. Platelets express caspase-8 and -10, but caspase-12 has not been detected in platelets, despite it is highly expressed in mature megacaryocytes (Kerrigan et al., 2004). As shown in Figure 2B, platelet incubation with the inhibitors of caspase-8 (Z-IETD-FMK; 100μM) or caspase-10 (Z-AEVD-FMK; 100 μM) had no significant effects on early caspase-3 activation stimulated by thrombin 0.01 or 1 U/ml.

Early caspase-3 activation is independent of rises in $[Ca^{2+}]_c$ but requires PKC activity

As mentioned above, activation of the thrombin receptor induces Ca^{2+} mobilization (Rink and Sage, 1990). Here, we have shown that depletion of the intracellular Ca^{2+} stores using TG + Iono was sufficient to induce activation of caspases-3 and -9 in platelets (Fig. 1B). In order to investigate whether this event is mediated by the elevation of $[Ca^{2+}]_c$, we loaded cell with dimethyl BAPTA, an intracellular Ca^{2+} chelator, by incubating the cells for 30 min with 10 μM dimethyl BAPTA AM. As shown in Figure 3A, dimethyl BAPTA-loading prevented thrombin-evoked $[Ca^{2+}]_c$ elevations. In contrast, BAPTA-loading had a negligible effect on thrombin-stimulated caspases-3 and -9 activation either at 1 or 60 min (Fig. 3B,C; $n = 6$). A similar effect was observed when caspase activation was stimulated by store depletion using TG + Iono (Fig. 3B,C), which suggests that depletion of the intracellular Ca^{2+} stores, rather than the subsequent increase in $[Ca^{2+}]_c$, is the mechanism involved in caspase activation. In addition to increasing the $[Ca^{2+}]_c$, activation of PLC by thrombin promotes the production of diacylglycerol, which in turn activates PKC. We next attempted to determine whether thrombin-stimulated activation of PKC was needed for its ability to cause caspase activation by testing the effect of the PKC inhibitor, Ro-31-8220 (Dieter and Fitzke, 1991). Pretreatment of platelets with 3 μM Ro-31-8220 for 5 min significantly reduced caspase-3 activation induced by stimulation for 1 min with 0.01 or 1 U/ml thrombin (Fig. 3D,E; $P < 0.05$; $n = 6$).

Role of early caspase-3 activation in thrombin-induced Ca^{2+} entry and platelet aggregation

Since early caspase-3 activation is induced by physiological concentrations of thrombin and is unlikely to be an apoptotic process we wondered whether this event might be involved cellular function. We have shown that activation of caspase-3 depends on Ca^{2+} store depletion but does not require rises in $[Ca^{2+}]_c$, a feature that is shared by store-operated Ca^{2+} entry (SOCE; Rosado et al., 2000a). Since SOCE is a major pathway for thrombin-induced Ca^{2+} entry, we have investigated the involvement of caspase-3 in this process. As we have previously shown (Rosado et al., 2004), treatment of platelets with thrombin in a Ca^{2+}-free medium results in a transient increase in $[Ca^{2+}]_c$ due to Ca^{2+} release from intracellular stores. Subsequent addition of Ca^{2+} to the extracellular medium induces a prolonged elevation in $[Ca^{2+}]_c$ indicative of SOCE (Fig. 4A). Treatment of platelets for 90 min at 37°C with 100 μM DEVD-CMK, a cell permeant inhibitor of caspase-3 (Wolf et al., 1999), significantly reduced Ca^{2+} entry evoked by thrombin at 0.01 or 1 U/ml by 25% and 40%, respectively, without

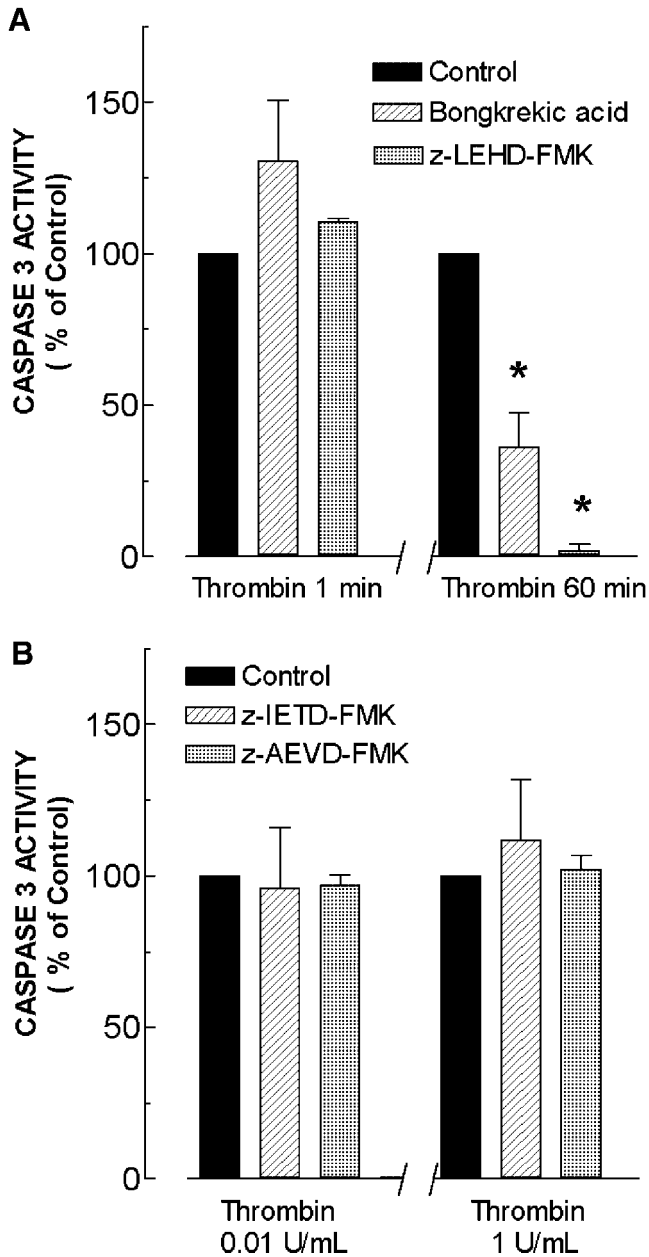

Fig. 2. Thrombin induces rapid activation of caspase-3 independently of caspase-8, -9, and -10. **A**: Human platelets were preincubated at 37°C either for 45 min with 50 μM bongkrekic acid or for 90 min with 40 μM z-LEHD-FMK (caspase-9 inhibitor) or the vehicle, and then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 1 U/ml thrombin for 1 min or 60 min, as indicated. Samples were analyzed following the caspase activity assay as described under Material and Methods. Values are presented as means ± SE (n = 6) and expressed as the percentage of Control (the increase caused by 1 U/ml thrombin, in the absence of inhibitors, above resting values). Asterisk indicates $P < 0.05$. **B**: Human platelets were preincubated at 37°C either for 90 min with 100 μM Z-IETD-FMK (caspase-8 inhibitor), 100 μM Z-AEVD-FMK (caspase-10 inhibitor), or the vehicle and then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 0.01 or 1 U/ml thrombin for 1 min, as indicated. Samples were analyzed following the caspase activity assay as described under Material and Methods. Values are presented as mean ± SE (n = 6) and expressed as the percentage of Control (the increase caused by thrombin, in the absence of inhibitors, above resting values).

having any significant effect on thrombin-stimulated Ca^{2+} release (Fig. 4A,B; $P < 0.05$; n = 6), which indicates that the effect of DEVD-CMK is specific for Ca^{2+} entry over Ca^{2+} release. These findings suggest that early caspase-3 activation by thrombin is at least partially required for the activation of Ca^{2+} entry. In contrast, treatment of platelets with the caspase-9 inhibitor, z-LEHD-FMK, had no significant effects on thrombin-evoked Ca^{2+} release or entry (Fig. 4C,D), which indicates that activation of caspase-9 is not involved in thrombin-evoked Ca^{2+} influx and is consistent with the apoptosis-related activation of caspase-9 reported above.

To obtain further insight into the role of early activation of caspase-3 on cell function, we investigated the involvement of caspase-3 in thrombin-induced platelet aggregation. As shown in Figure 4E,F *control*, treatment of human platelets with 0.01 or 1 U/ml thrombin induced an initial shape change and then a rapid aggregation. In platelets preincubated with the caspase-3 inhibitor, DEVD-CMK, aggregation occurred slower and the amplitude was smaller (41.3 ± 4.1 and 48.2 ± 2.5% in DEVD-CMK-treated cells and 67 ± 5.1 and 73.7 ± 3.4% in controls, for 0.01 and 1 U/ml thrombin, respectively; Fig. 4E,F; $P < 0.01$; n = 15–28). These findings strongly suggest that thrombin-induced early caspase-3 activation is required for platelet function. The lack of PS exposure upon treatment with 0.01 U/ml thrombin, at least for 1 h (Fig. 1E), suggests that this effect is independent of apoptosis.

Rapid caspase-3 activation is required for thrombin-induced activation of the tyrosine kinases Btk and pp60^{src}

Tyrosine kinases have been shown to play an essential role in the activation of a number of thrombin-stimulated cellular processes, including SOCE (Rosado et al., 2000a) and platelet aggregation (Feinstein et al., 1993; Canobbio et al., 2004). At present, the only tyrosine kinase proteins that have been identified as components of SOCE are the cytosolic proteins Btk and pp60^{src} (Babnigg et al., 1997; Rosado et al., 2000a; Redondo et al., 2003, 2005b). Hence, we have investigated the role of caspase-3 in the activation of Btk and pp60^{src}.

The activation of Btk was analyzed by Western blotting using a rabbit monoclonal phosphospecific anti-Btk antibody that only detects Btk autophosphorylated at the tyrosine residue 223, which has been shown to be the full activated form of Btk (Rawlings et al., 1996). Treatment of human platelets in a Ca^{2+}-free medium with 1 U/ml thrombin or by store depletion using TG + Iono caused rapid and time-dependent activation of Btk (Fig. 5, *top and bottom parts*). Preincubation of platelets for 90 min at 37°C with 100 μM DEVD-CMK, the inhibitor of caspase-3, signifcantly reduced agonist-induced activation of Btk without having significant effects on autophosphorylation level of Btk in non-stimulated platelets (Fig. 5; $P < 0.001$; n = 4).

Similar results were obtained when the involvement of caspase-3 on pp60^{src} activity was investigated. The activation of pp60^{src} was investigated by Western blotting using a specific anti-phospho-c-Src (Y-416) antibody, which specifically detects pp60^{src} phosphorylated at Tyr-416, a process indicative of its activation (Osusky et al., 1995). As shown in Figure 6, pretreatment of platelets for 90 min at 37°C with the caspase-3 inhibitor, DEVD-CMK (100 μM), significantly reduced agonist-induced activation of pp60^{src} (Fig. 6; $P < 0.001$; n = 4). As for Btk, DEVD-CMK induced no significant effects on basal phosphotyrosine levels of pp60^{src} (Fig. 6; n = 4).

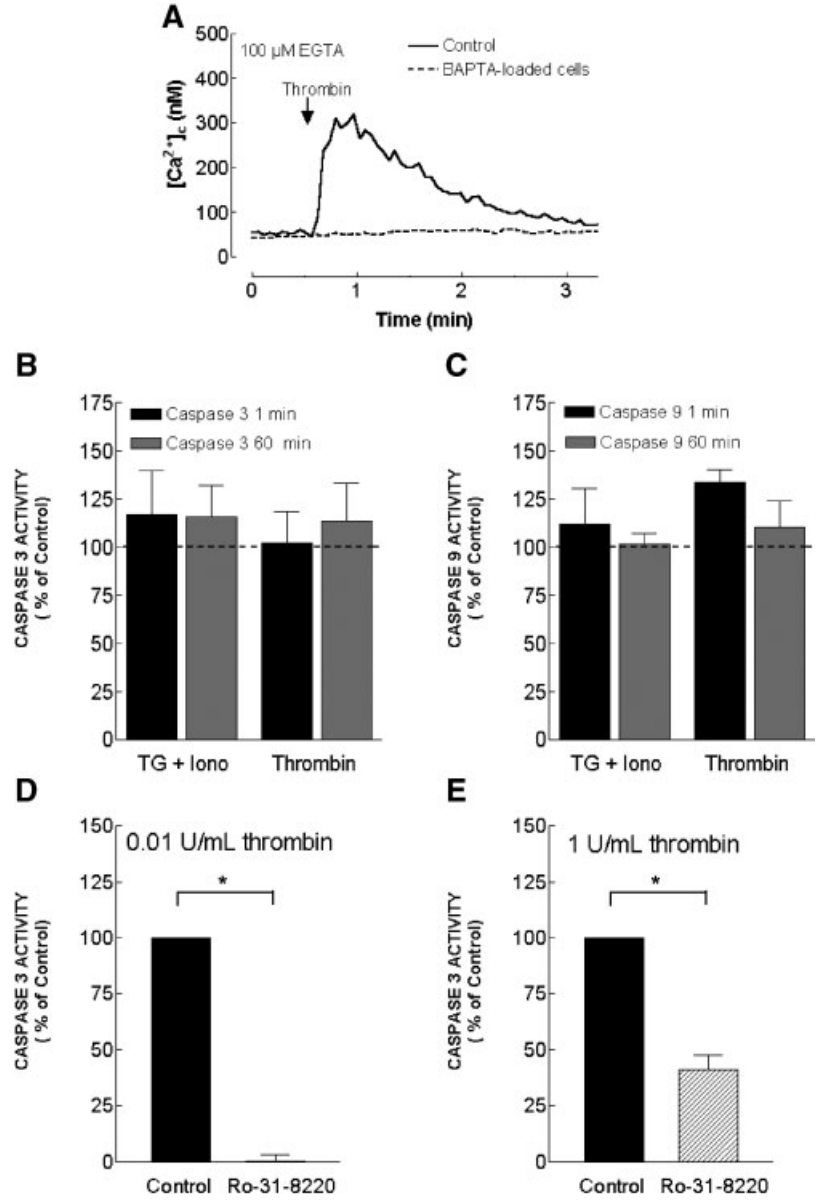

Fig. 3. Protein kinase C but not rises in $[Ca^{2+}]_c$ are required for early activation of caspase-3. **A**: Fura 2-loaded human platelets were incubated at 37°C for 30 min in the presence or absence (Control) of 10 μM dimethyl BAPTA. At the time of experiment 100μM EGTA was added, as indicated, and cells were then stimulated with 1 U/ml thrombin. Elevations in $[Ca^{2+}]_c$ were monitored using the 340/380 nm ratio and traces were calibrated in terms of $[Ca^{2+}]_c$ as described under Material and Methods. Traces shown are representative of five independent experiments. **B** and **C**: Human platelets were loaded with dimethyl BAPTA as described above and stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with TG (1 μM) + Iono (50 nM) or with thrombin (1 U/ml) for 1 min or 60 min. Caspase-3 (**B**) and caspase-9 (**C**) activities were analyzed following the caspase activity assay as described under Material and Methods. Values are presented as mean ± SE (n = 6) and expressed as the percentage of Control (the increase caused by TG + Iono or thrombin, in the absence of dimethyl BAPTA, above resting values, *dashed lines*). **D** and **E**: platelets were preincubated at 37°C for 5 min with 3 μM Ro-31-8220 or the vehicle and then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 0.01 U/ml (**D**) or 1 U/ml (**E**) thrombin for 1 min. Caspase-3 activity was determined as described under Material and Methods and values are presented as mean ± SE (n = 6) expressed as the percentage of Control (the increase caused by thrombin, in the absence of Ro-31-8220, above resting values). Asterisk indicates $P < 0.05$.

Early caspase-3 activation is required for CCK-8-stimulated secretion in pancreatic acinar cells

To further investigate whether the requirement of agonist-induced early activation of caspase-3 for cellular physiology is a general process in mammalian cells we performed a series of experiments in mouse pancreatic acinar cells, a nucleated cell model. Our results indicate that stimulation of pancreatic acinar cells with the physiological agonist CCK-8 (1 nM) induces rapid activation of caspase-3 that was detected within 1 min of stimulation with a 3.8 ± 0.5-fold increase and then returned to basal at 10 min with a 1.5 ± 0.4-fold increase (Fig. 7A). Recent studies have reported that prolonged (3 h) stimulation of pancreatic acinar cells with CCK-8, at concentrations between 1 and 100 nM, stimulates apoptosis and death signaling pathways, including caspase-3 activation (Gukovskaya et al., 2002), which indicates that, as in platelets, activation of caspase-3 by CCK-8 in pancreatic acinar cells is bimodal. A more physiological concentration of CCK-8 (30 pM), which has been determined in plasma in response to food intake (Chey, 1986), have been also found to increase caspase 3 activity after 1 min of stimulation. Early activation of

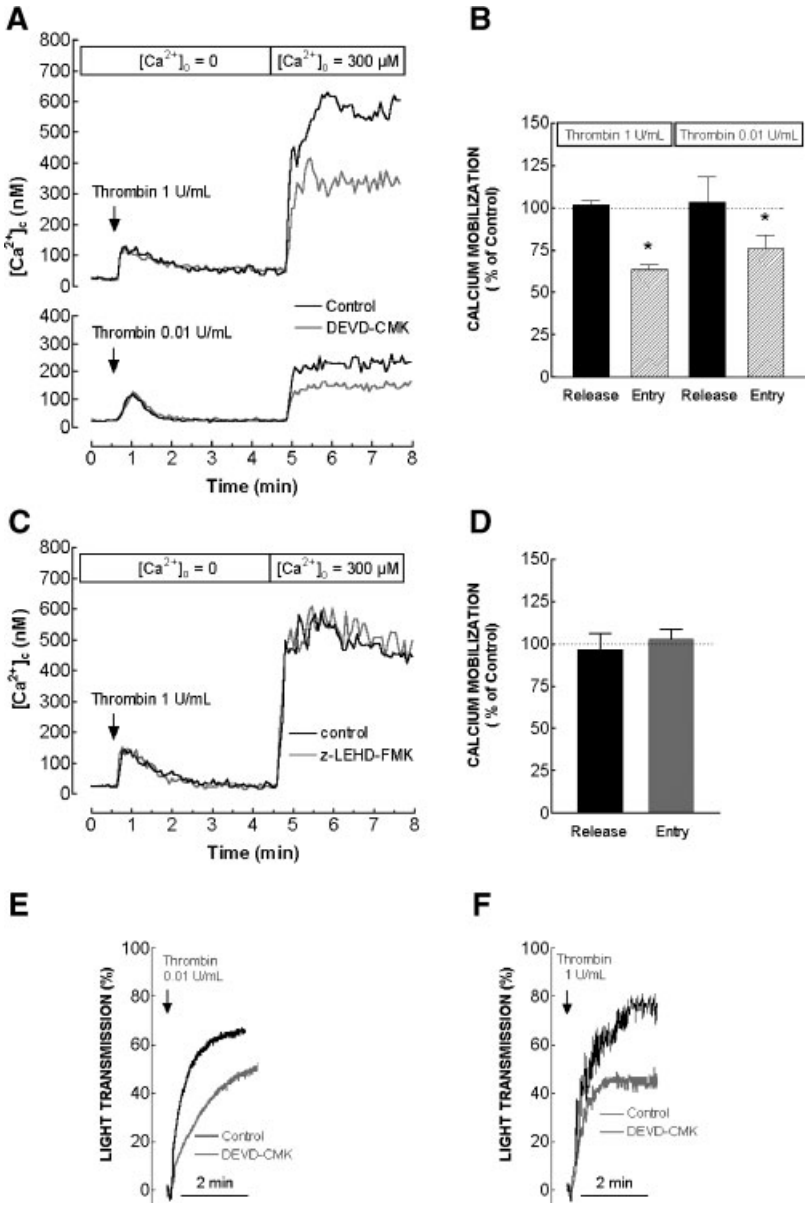

Fig. 4. Early activation of caspase-3 is required for the activation of SOCE and platelet aggregation. **A** and **B**: Fura-2-loaded human platelets were preincubated for 90 min at 37°C with 100 μM DEVD-CMK or the vehicle (DMSO) as Control. Cells were then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with thrombin (0.01 or 1 U/ml, as indicated) followed by addition of $CaCl_2$ (final concentration 300 μM) to initiate Ca^{2+} entry. Histograms indicate the percentage of Ca^{2+} mobilization (Ca^{2+} release or entry, as indicated) in the presence of DEVD-CMK relative to their respective Controls (vehicle was added). Ca^{2+} entry was determined as described under Material and Methods. Values are mean ± SE (n = 6). Asterisk indicates $P < 0.05$. **C** and **D**: Fura-2-loaded human platelets were preincubated for 90 min at 37°C with 40 μM z-LEHD-FMK or the vehicle (DMSO) as Control. Cells were then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with thrombin (1 U/ml) followed by addition of $CaCl_2$ (final concentration 300 μM) to initiate Ca^{2+} entry. Histograms indicate the percentage of Ca^{2+} mobilization (Ca^{2+} release or entry, as indicated) in the presence of z-LEHD-FMK relative to its Control (vehicle was added). Ca^{2+} entry was determined as described under Material and Methods. Values are means ± SE (n = 6). **E** and **F**: Cells were preincubated for 90 min at 37°C with 40 μM DEVD-CMK or the vehicle (DMSO) as Control. Aggregation of washed platelets was produced for 3 min at a shear rate of 1,200 rpm in an aggregometer by thrombin (0.01 U/ml (E) or 1 U/ml (F)), added as indicated.

caspase-3 by CCK-8 was significantly smaller than that observed after treatment for 2 h with 1 μM TG (Fig. 7A; n = 6), which can induce apoptosis in a wide variety of epithelial and non-epithelial cell lineages (Baffy et al., 1993; Qi et al., 1997). The observations that early caspase-3 activation is smaller than that induced during apoptosis is common for platelets and pancreatic acinar cells. In order to investigate whether this event is related with the initial phase of apoptosis we investigated PS exposure after stimulation with different concentrations of CCK-8. Treatment of pancreatic acinar cells for 1 min with CCK-8 (30 pM–1 nM) induces a concentration-dependent incrase in PS exposure (Fig. 7B; n = 6). PS externalization was not detected at 30 pM CCK-8 either at 1 min (Fig. 7B) or after 30 min of stimulation (PS exposure after 30 min treatment with CCK-8 30 pM was 0.61 ± 0.14-fold under control; n = 6). We have further investigated the involvement of caspase-3 in pancreatic acinar cell function by testing the effect of DEVD-CMK on amylase secretion evoked by CCK-8. In mouse pancreatic acinar cells incubated with increasing concentrations of CCK-8, amylase release was detectable with 10 pM CCK-8, half maximal with 100 pM CCK-8, and maximal with 300 pM, with

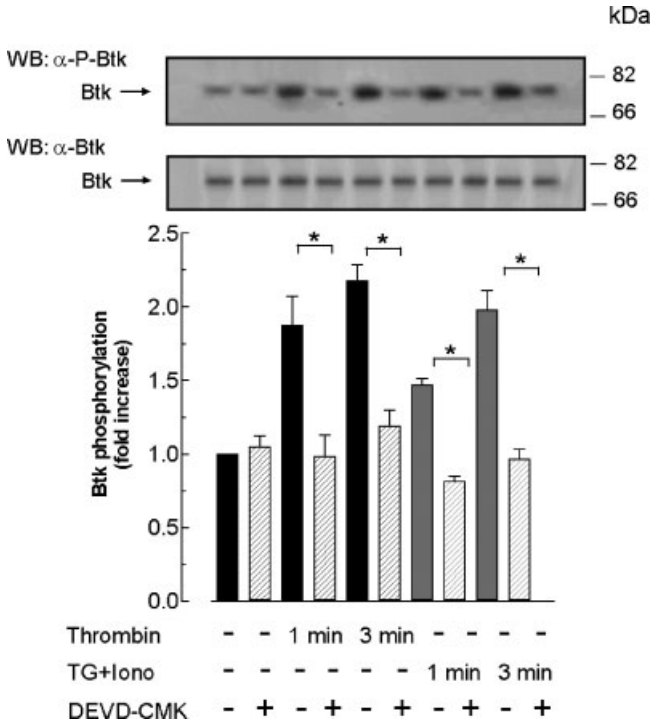

Fig. 5. Caspase-3 activity is required for thrombin- and TG + Iono-induced activation of Btk. Human platelets were preincubated for 90 min at 37°C with 100 μM DEVD-CMK or the vehicle (DMSO) as control and then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 1 U/ml thrombin or 1 μM TG + 50 nM Iono for a further 1 or 3 min as indicated and lysed. Samples were subjected to SDS/PAGE and Western blotting with the specific anti phospho-Btk (Y-223; α-P-Btk) antibody or the anti-Btk antibody (α-Btk), for protein loading control, as described under Material and Methods. Bands were revealed using chemiluminescence and were quantified using scanning densitometry. The upper parts show results from one experiment representative of three others. Positions of molecular-mass markers are shown on the right. The bottom part shows the quantification of Btk phosphorylation at the tyrosine residues 223. Values are means ± SE from four separate experiments expressed as fold increase over the pretreatment level. *$P < 0.001$ compared to control.

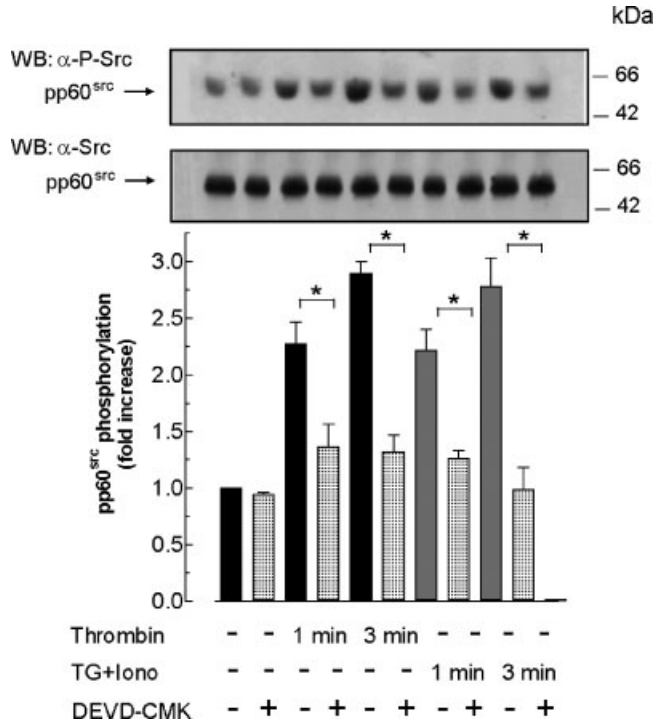

Fig. 6. Caspase-3 activity is required for thrombin- and TG + Iono-induced activation of $pp60^{src}$. Human platelets were preincubated for 90 min at 37°C with 100 μM DEVD-CMK or the vehicle (DMSO) as control and then stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with 1 U/ml thrombin or 1 μM TG + 50 nM Iono for a further 1 or 3 min as indicated and lysed. Samples were subjected to SDS/PAGE and Western blotting with the specific anti-phospho-c-Src (Y-416; α-P-Src) antibody or the anti-$pp60^{src}$ antibody (α-Src), for protein loading control, as described under Material and Methods. Bands were revealed using chemiluminescence and were quantified using scanning densitometry. The upper parts show results from one experiment representative of three others. Positions of molecular-mass markers are shown on the right. The bottom part shows the quantification of $pp60^{src}$ phosphorylation at the tyrosine residues 416. Values are mean ± SE from four separate experiments expressed as fold increase over the pretreatment level. *$P < 0.001$ compared to control.

8.8 ± 0.6% of total over basal, and then decreased by up to approximately 60% with supramaximal concentrations of CCK-8 (Fig. 7C). Treatment of pancreatic acinar cells for 90 min with 100 μM DEVD-CMK did not significantly modify the basal levels of amylase release (basal values were 5.0 ± 0.5 and 6.9 ± 0.9% of total for cells incubated in the absence and presence of DEVD-CMK, respectively) but clearly reduced amylase release stimulated by CCK-8 (Fig. 7C; $P < 0.05$; n = 6). As reported above, cell treatment with 30 pM CCK-8 for 30 min, the incubation time established to determine amylase secretion (see Material and Methods), induced caspase-3 activation independently of the development of apoptosis, as detected by PS exposure (Fig. 7B). The effect of the caspase-3 inhibitor on amylase release by 30 pM CCK-8 suggests that caspase-3 activation is involved in the physiological activation of amylase secretion in pancreatic acinar cells.

DISCUSSION

Caspase-3 is a major mediator of apoptotic cell death and its activation has widely been considered as an apoptotic marker. We show that thrombin induces a sequential and bimodal activation of caspase-3 at concentrations as low as 0.01 U/ml. The early activation of caspase-3 occurs independently of caspases-8, -9, and -10 and mitochondrial-dependent apoptosis, while delayed caspase-3 activation is more likely an apoptotic event dependent on caspase-9 and mitochondrial cytochrome c release. These results were confirmed by investigating the ability of thrombin to induce PS exposure, which paralleled the activation of caspase-9. We provide evidences supporting that thrombin-induced early caspase-3 activation is required for agonist activated cellular functions in two non-related cell models.

We report that early caspase-3 activation induced by treatment for 1 min with thrombin at low concentrations (0.01 U/ml) or 1 U/ml, which were unable to evoke PS exposure, requires PKC activity in human platelets. PKC has been previously shown to amplify apoptotic signaling via activation of the caspase cascade (see Kanthasamy et al., 2003); however, inhibition of PKC has also been reported to attenuate apoptosis induced by different factors (Imamdi et al., 2004; Zhou et al., 2005). To our knowledge, we provide the first description of a role for PKC in non-apoptotic activation of caspase-3.

Ca^{2+} has been presented as a key regulator of cell survival but this ion can also induce apoptosis in response to a number of pathological conditions (see Hajnoczky et al., 2003; Orrenius et al., 2003). $[Ca^{2+}]_c$ elevation has been demonstrated to play a critical role in

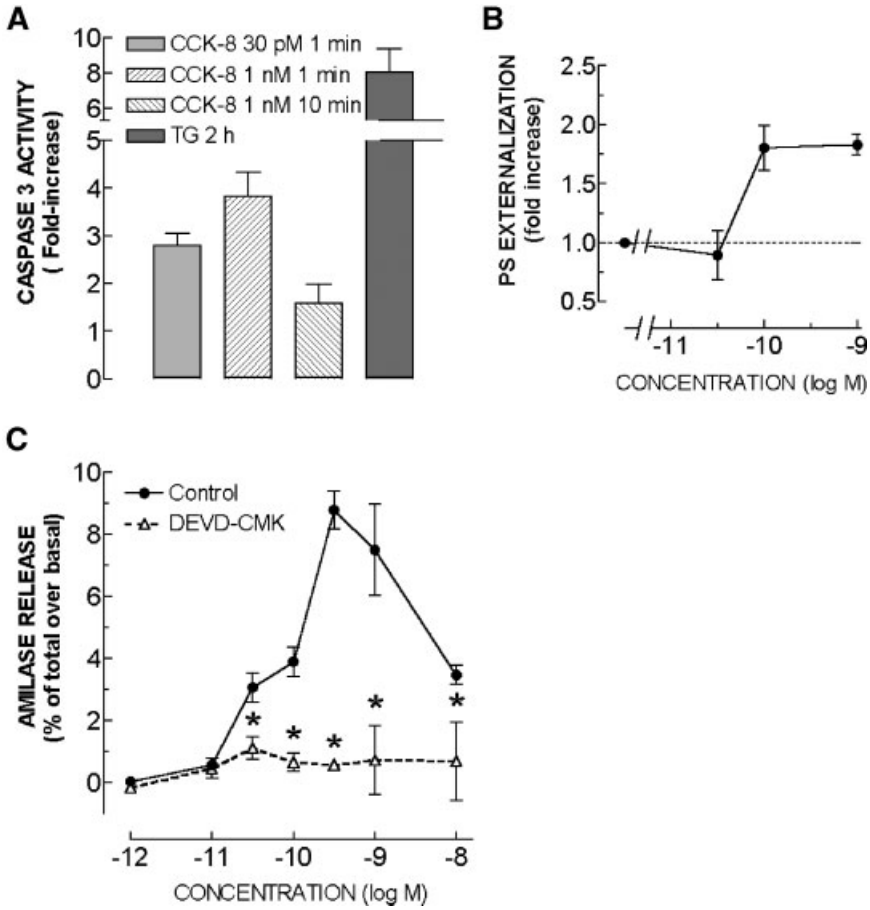

Fig. 7. CCK-8 induces rapid activation of caspase-3 that is required for amylase secretion. **A**: Freshly isolated pancreatic acinar cells were stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with CCK-8 30 pM for 1 min, CCK-8 1 nM for 1 or 10 min, or with TG (1 μM) for 2 h. Caspase-3 activity was analyzed following the caspase activity assay as described under Material and Methods. Values are presented as mean ± SE (n = 6) expressed as the percentage of resting values. **B**: Pancreatic acinar cells were stimulated in a Ca^{2+}-free medium (100 μM EGTA was added) with CCK-8 (30 pM–1 nM) for 1 min. PS externalization was analyzed as described under Material and Methods. Values are presented as means ± SE (n = 6) expressed as fold-increase over the pretreatment level (experimental/control). **C**: Pancreatic acinar cells were incubated for 90 min with 100 μM DEVD-CMK or the vehicle (DMSO) as Control and then were stimulated for 30 min at 37°C with different concentrations of CCK-8 as indicated in a medium HBS containing 1.3 mM $CaCl_2$. Acini were then pelleted and amylase activity in the supernatant was determined as described under Material and Methods. Amylase activity was expressed as the percentage of cellular amylase released into the extracellular medium during the incubation and presented as the increase over basal. Basal values were 5.0 ± 0.5 and 6.9 ± 0.9% of total for cells incubated in the absence and presence of DEVD-CMK, respectively. Results are mean ± SE of six separate experiments in which each point was determined in duplicate. Asterisk indicates $P < 0.05$ compared with amylase release in cells stimulated with CCK-8 in the absence of the inhibitor.

regulating the activity of caspases-3 and -9 (Tantral et al., 2004). We now show that thrombin induced caspase-3 and -9 activation independently of rises in $[Ca^{2+}]_c$. Similar results were observed after depletion of the intracellular Ca^{2+} stores, using TG in combination with Iono, in BAPTA-loaded cells, which efficiently prevents $[Ca^{2+}]_c$ elevations, suggesting that agonist-induced store depletion rather than the subsequent rise in $[Ca^{2+}]_c$, is responsible for the activation of both caspases in platelets. The lack of dependence of rises in $[Ca^{2+}]_c$ resembles the mechanisms involved in the activation of SOCE in platelets (Sargeant et al., 1994), where a number of intracellular messengers, including the cytosolic tyrosine kinases Btk and $pp60^{src}$, have been shown to be involved in the de novo conformational coupling between TRPC1 and IP_3 receptor type II (Redondo et al., 2005b; Rosado et al., 2005). Our results demonstrate that activation of caspase-3 by concentrations as low as 0.01 U/ml thrombin, supposedly the early activation, is required for the activation of both Btk and $pp60^{src}$, and subsequently for SOCE in human platelets. We have previously shown that depletion of the intracellular stores in platelets is accompanied by Ca^{2+}-independent H_2O_2 generation (Rosado et al., 2004) and H_2O_2-mediated oxidative stress has been reported to induce the activation of PKC, as well as caspase-3, Btk, and $pp60^{src}$ (Ueda et al., 2002; Rosado et al., 2004; Redondo et al., 2005b; Ryer et al., 2005). Although speculative, H_2O_2 generated upon Ca^{2+} store depletion might induce the activation of caspase-3 by PKC. PKC isoforms might also be involved in the activation of Btk and $pp60^{src}$ by caspase-3, suggesting a role for PKC both in the activation of caspase-3 and in caspase-3-mediated effects. This hypothesis is based on previous findings reporting both, a reciprocal interaction between Btk and PKC_0 in platelets, in which PKC_0 positively modulates Btk activity, which in turn feeds back negatively upon PKC_0 (Crosby and Poole, 2002), and a role for PKC in the activation of $pp60^{src}$ by H_2O_2 and physiological agonists (Rosado et al., 2004).

SOCE is a process required for full activation of a number of cellular processes including platelet aggregation (Merritt et al., 1990) and pancreatic enzyme secretion (Tsunoda et al., 1990). Consistent with this, we show that platelet aggregation and pancreatic amylase secretion stimulated by physiological agonists

are sensitive to inhibition of caspase-3. Our results are consistent with a physiological role of early caspase-3 activation in cell function since the involvement of caspase-3 in calcium signaling and platelet aggregation induced by 0.01 U/ml thrombin is independent of apoptosis. Thrombin induced early activation of caspase-3 at a range of concentrations similar to that found to activate physiological processes, including Ca^{2+} release and aggregation (Jakubowski and Maraganore, 1990; Heemskerk et al., 1997), and, as many physiological processes, early activation of caspases-3 is reduced by supramaximal concentrations of agonists (Niederau et al., 1994). In contrast, delayed caspase-3 activation is more likely an apoptotic event, since it is sensitive to inhibitors of both, caspase-9 activation and cytochrome c release, and it is maximally activated by supramaximal concentrations of agonists. Therfore, our findings suggest a dual role for caspase-3 activation depending on the time of activation and the concentration of the agonist. Caspase-3 activation by low (physiological) concentrations of agonists or the early component of caspase-3 activation appears to be involved in physiological processes, while late, secondary to caspase-9 function, activation of caspase-3 is likely to be involved in the development of apoptosis.

Accumulating evidence suggest that, although high agonist concentrations induce early and late caspase-3 activation, the latter might be involved in apoptosis, agonists at physiological concentrations induce rapid caspase-3 activation independently of the apoptotic pathway that includes mitochondrial cytochrome c release, and the subsequent activation of caspase-9 and PS exposure. Early caspase-3 activation was smaller than that observed during the development of apoptosis and was achieved by agonist concentrations reported to be involved in the stimulation of physiological processes. Finally, early caspase-3 activation by agonists have been shown to be required for full activation of a number of cellular functions, including aggregation and secretion. We propose that the involvement of early activation of caspase-3 in cellular function is likely a general event in mammalian cells.

ACKNOWLEDGMENTS

This work was supported by MCyT-DGI grant BFU2004-00165. J.J.L. is supported by a DGI fellowship (BFU2004-00165). We thank Ana M. Nuñez and Mercedes Gómez Blázquez for their technical assistance.

LITERATURE CITED

Ashkenazi A, Dixit VM. 1998. Death receptors: Signaling and modulation. Science 281:1305–1308.

Babnigg G, Bowersox SR, Villereal ML. 1997. The role of pp60c-src in the regulation of calcium entry via store-operated calcium channels. J Biol Chem 272:29434–29437.

Baffy G, Miyashita T, Williamson JR, Reed JC. 1993. Apoptosis induced by withdrawal of interleukin-3 (IL-3) from an IL-3-dependent hematopoietic-cell line is associated with repartioning of intracellular calcium and is blocked by enforced bcl-2 oncoprotein production. J Biol Chem 268:6511–6519.

Ben Amor N, Pariente JA, Salido GM, Bartegi A, Rosado JA. 2006. Caspases 3 and 9 are translocated to the cytoskeleton and activated by thrombin in human platelets. Evidence for the involvement of PKC and the actin filament polymerization. Cell Signal 18:1252–1261.

Canobbio I, Balduini C, Torti M. 2004. Signalling through the platelet glycoprotein Ib-V-IX complex. Cell Signal 16:1329–1344.

Cavallini L, Coassin M, Alexandre A. 1995. Two classes of agonist-sensitive Ca^{2+} stores in platelets, as identified by their differential sensitivity to 2,5-di-(tert-butyl)-1,4-benzohydroquinone and thapsigargin. Biochem J 310:449–452.

Chey WY. 1986. Hormonal control of pancreatic exocrine secretion. In: Go VLW, editor. The exocrine pancreas: Biology, pathobiology and diseases. New York: Raven Press. pp 301–313.

Crosby D, Poole AW. 2002. Interaction of Bruton's tyrosine kinase and protein kinase Ctheta in platelets. Cross-talk between tyrosine and serine/threonine kinases. J Biol Chem 277:9958–9965.

Dieter P, Fitzke E. 1991. RO 31-8220 and RO 31-7549 show improved selectivity for protein kinase C over staurosporine in macrophages. Biochem Biophys Res Commun 181:396–401.

Feinstein MB, Pumiglia K, Lau LF. 1993. Tyrosine phosphorylation in platelets: Its regulation and possible roles in platelet functions. Adv Exp Med Biol 344:129–148.

Gerasimenko JV, Gerasimenko OV, Palejwala A, Tepikin AV, Petersen OH, Watson AJ. 2002. Menadione-induced apoptosis: Roles of cytosolic Ca^{2+} elevations and the mitochondrial permeability transition pore. J Cell Sci 115:485–497.

Green DR, Reed JC. 1998. Mitochondria and apoptosis. Science 281:1309–1312.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Gukovskaya AS, Gukovsky I, Jung Y, Mouria M, Pandol SJ. 2002. Cholecystokinin induces caspase activation and mitochondrial dysfunction in pancreatic acinar cells. Roles in cell injury processes of pancreatitis. J Biol Chem 277:22595–22604.

Hajnoczky G, Davies E, Madesh M. 2003. Calcium signaling and apoptosis. Biochem Biophys Res Commun 304:445–454.

Heemskerk JW, Feijge MA, Henneman L, Rosing J, Hemker HC. 1997. The Ca^{2+}-mobilizing potency of alpha-thrombin and thrombin-receptor-activating peptide on human platelets. Concentration and time effects of thrombin-induced Ca^{2+} signaling. Eur J Biochem 249:547–555.

Imamdi R, de Graauw M, van de Water B. 2004. Protein kinase C mediates cisplatin-induced loss of adherens junctions followed by apoptosis of renal proximal tubular epithelial cells. J Pharmacol Exp Ther 311:892–903.

Ishizaki Y, Jacobson MD, Raff MC. 1998. A role for caspases in lens fiber differentiation. J Cell Biol 140:153–158.

Jakubowski JA, Maraganore JM. 1990. Inhibition of coagulation and thrombin-induced platelet activities by a synthetic dodecapeptide modeled on the carboxy-terminus of hirudin. Blood 75:399–406.

Kanthasamy AG, Kitazawa M, Kanthasamy A, Anantharam V. 2003. Role of proteolytic activation of protein kinase Cdelta in oxidative stress-induced apoptosis. Antioxid Redox Signal 5:609–620.

Kerrigan SW, Gaur M, Murphy RP, Shattil SJ, Leavitt AD. 2004. Caspase-12: A developmental link between G-protein-coupled receptors and integrin alphaIIbbeta3 activation. Blood 104:1327–1334.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang XD. 1997. Cytocrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

Merritt JE, Armstrong WP, Benham CD, Hallam TJ, Jacob R, Jaxa-Chamiec A, Leigh BK, McCarthy SA, Moores KE, Rink TJ. 1990. SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem J 271: 515–522.

Niederau C, Luthen R, Heintges T. 1994. Effects of CCK on pancreatic function and morphology. Ann NY Acad Sci 713:180–198.

Orrenius S, Zhivotovsky B, Nicotera P. 2003. Regulation of cell death: The calcium-apoptosis link. Nat Rev Mol Cell Biol 4:552–565.

Osusky M, Taylor SJ, Shalloway D. 1995. Autophosphorylation of purified c-Src at its primary negative regulation site. J Biol Chem 270:25729–25732.

Ozoren N, Kim K, Burns TF, Dicker DT, Moscioni AD, El-Deiry WS. 2000. The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. Cancer Res 60:6259–6265.

Qi XM, He H, Zhong H, Distelhorst CW. 1997. Baculovirus p35 and Z-VAD-fmk inhibit thapsigargin-induced apoptosis of breast cancer cells. Oncogene 15:1207–1212.

Rawlings DJ, Scharenberg AM, Park H, Wahl MI, Lin S, Kato RM, Fluckiger AC, Witte ON, Kinet JP. 1996. Activation of BTK by a phosphorylation mechanism initiated by SRC family kinases. Science 271:822–825.

Redondo PC, Lajas AI, Salido GM, Gonzalez A, Rosado JA, Pariente JA. 2003. Evidence for secretion-like coupling involving pp60src in the activation and maintenance of store-mediated Ca^{2+} entry in mouse pancreatic acinar cells. Biochem J 370:255–263.

Redondo PC, Jardin I, Hernandez-Cruz JM, Pariente JA, Salido GM, Rosado JA. 2005a. Hydrogen peroxide and peroxynitrite enhance Ca^{2+} mobilization and aggregation in platelets from type 2 diabetic patients. Biochem Biophys Res Commun 333:794–802.

Redondo PC, Ben-Amor N, Salido GM, Bartegi A, Pariente JA, Rosado JA. 2005b. Ca^{2+}-independent activation of Bruton's tyrosine kinase is required for store-mediated Ca^{2+} entry in human platelets. Cell Signal 17:1011–1021.

Rink TJ, Sage SO. 1990. Calcium signalling in human platelets. Annu Rev Physiol 52:431–439.

Rosado JA, Sage SO. 2000a. Farnesylcysteine analogues inhibit store-regulated Ca^{2+} entry in human platelets: Evidence for involvement of small GTP-binding proteins and actin cytoskeleton. Biochem J 346:183–192.

Rosado JA, Sage SO. 2000b. Coupling between inositol 1,4,5-trisphosphate receptors and human transient receptor potential channel 1 when intracellular Ca^{2+} stores are depleted. Biochem J 350:631–635.

Rosado JA, Salido GM, Jensen RT, Garcia LJ. 1998. Are tyrosine phosphorylation of $p125^{FAK}$ and paxillin or the small GTP binding protein, rho, needed for CCK-stimulated pancreatic amylase secretion? Biochim Biophys Acta 1404:412–426.

Rosado JA, Graves D, Sage SO. 2000a. Tyrosine kinases activate store-mediated Ca^{2+} entry in human platelets through the reorganization of the actin cytoskeleton. Biochem J 351:429–437.

Rosado JA, Jenner S, Sage SO. 2000b. A role for the actin cytoskeleton in the initiation and maintenance of store-mediated calcium entry in human platelets. Evidence for conformational coupling. J Biol Chem 275:7527–7533.

Rosado JA, Redondo PC, Salido GM, Gomez-Arteta E, Sage SO, Pariente JA. 2004. Hydrogen peroxide generation induces $pp60^{src}$ activation in human platelets: Evidence for the involvement of this pathway in store-mediated calcium entry. J Biol Chem 279:1665–1675.

Rosado JA, Redondo PC, Sage SO, Pariente JA, Salido GM. 2005. Store-operated Ca^{2+} entry: Vesicle fusion or reversible trafficking and de novo conformational coupling? J Cell Physiol 205:262–269.

Ryer EJ, Sakakibara K, Wang C, Sarkar D, Fisher PB, Faries PL, Kent KC, Liu B. 2005. Protein kinase C delta induces apoptosis of vascular smooth muscle cells through induction of the tumor suppressor p53 by both p38-dependent and p38-independent mechanisms. J Biol Chem 280:35310–35317.

Sargeant P, Farndale RW, Sage SO. 1994. Calcium store depletion in dimethyl BAPTA-loaded human platelets increases protein tyrosine phosphorylation in the absence of a rise in cytosolic calcium. Exp Physiol 79:269–272.

Shcherbina A, Remold-O'Donnel E. 1999. Role of caspase in a subset of human platelet activation responses. Blood 93:4222–4231.

Shi Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis. Mol Cell 9:459–470.

Stennicke HT, Salvesen GS. 1998. Biochemical characteristics of caspases-3, -6, -7, and -8. J Biol Chem 272:25719–25723.

Tantral L, Malathi K, Kohyama S, Silane M, Berenstein A, Jayaraman T. 2004. Intracellular calcium release is required for caspase-3 and -9 activation. Cell Biochem Funct 22:35–40.

Tsunoda Y, Stuenkel EL, Williams JA. 1990. Characterization of sustained $[Ca^{2+}]_i$ increase in pancreatic acinar cells and its relation to amylase secretion. Am J Physiol 259:G792–G801.

Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, Yodoi J. 2002. Redox control of cell death. Antioxid Redox Signal 4:405–414.

Wilhelm S, Wagner H, Häcker G. 1998. Activation of caspase-3-like enzymes in non-apoptotic T cells. Eur J Immunol 28:891–900.

Wolf BB, Goldstein JC, Stennicke HR, Beere H, Amarante-Mendes GP, Salvesen GS, Green DR. 1999. Calpain functions in a caspase-independent manner to promote apoptosis-like events during platelet activation. Blood 94:1683–1692.

Zhou Y, Wang Q, Evers BM, Chung DH. 2005. Signal transduction pathways involved in oxidative stress-induced intestinal epithelial cell apoptosis. Pediatr Res 58:1192–1197.