Involvement of extracellular calcium in phosphatidylserine exposure during apoptosis

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Abstract The appearance of phosphatidylserine (PS) on the outer surface of apoptotic cells is an important signal for their ingestion. In platelets, elevation of intracellular Ca^{2+} with thapsigargin can trigger large amounts of PS exposure within minutes. We detected PS exposure in U937 promonocytes and Jurkat T-cells after incubation with thapsigargin, but in only 10% of the cells, and it took up to 6 h to occur. Tumor necrosis factor and anti-Fas antibody rapidly trigger apoptosis in these cells, and chelation of extracellular Ca^{2+} with 5 mM EGTA inhibited PS exposure by 65% and 50%, respectively. Chelation of intracellular Ca^{2+} with BAPTA-AM had no effect. Other parameters of apoptosis, including cell blebbing, shrinkage, nuclear fragmentation, activation of the ICE-like proteases, and fodrin cleavage, were not inhibited by extracellular EGTA. We conclude that while an elevation of intracellular Ca^{2+} is an ineffective trigger of apoptosis in the cells investigated, extracellular Ca^{2+} is required for efficient PS exposure during apoptosis.

Key words: Apoptosis; Phosphatidylserine; Ca^{2+}; Thapsigargin; Tumor necrosis factor; Anti-fas

1. Introduction

Cells undergoing apoptosis are ingested by both neighbouring cells and professional phagocytes. This prevents any inflammation and tissue damage that could occur upon lysis of the dying cell. One important mechanism for the recognition of apoptotic cells is the expression of phosphatidylserine (PS) on their outer surface [1]. PS is normally constrained to the inner leaflet of the plasma membrane [2], but disruption of this asymmetry has been reported in a variety of cell types undergoing apoptosis [3–9].

It has been known for many years that PS is exposed on the surface of activated platelets, where it plays an important role in the clotting cascade [10]. PS can also be exposed by red blood cells and is involved in their clearance from circulation [11]. Platelet activation causes rapid increase in intracellular Ca^{2+}, and pharmacological modulation with Ca^{2+} ionophores or Ca^{2+}-ATPase inhibitors can stimulate the exposure of large amounts of PS by platelets within minutes [12]. Ca^{2+} inactivates the aminophospholipid translocase which normally constrains PS to the inner leaflet [13], but this alone is insufficient for exposure. Another protein is proposed to mediate the scrambling of membrane phospholipids [14], although there is debate as to whether there is a general phospholipid scrambling or a specific translocation of PS [15]. Reconstituted membranes have been used to study these mechanisms [16], and recently a 37 kDa protein has been extracted from red cell ghosts that can transport labelled phospholipids upon triggering with Ca^{2+} [17].

A variety of studies implicate Ca^{2+} in the regulation of apoptosis [18]. Disruption of Ca^{2+} homeostasis can trigger apoptosis [19], an increase in the concentration of intracellular Ca^{2+} has been reported in some apoptotic models [20], and Ca^{2+} chelators have been observed to block apoptosis [21]. In other models, changes in intracellular Ca^{2+} are not so apparent, and Ca^{2+} appears to inhibit apoptosis [22].

As elevation of intracellular Ca^{2+} leads to rapid PS exposure in platelets, we tested the ability of thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase [23], to initiate PS exposure in Jurkat T-cells and U937 promonocytes. We also used chelators to investigate the involvement of Ca^{2+} in PS exposure upon stimulation of apoptosis in these cells with anti-Fas antibody and TNF.

2. Materials and methods

2.1. Materials

Jurkat and U937 cells were obtained from the Microbiology and Tumour Biology Centre, Karolinska Institutet. TNF was a generous gift from Dr. Grace Wong (Genentech Inc, San Francisco, CA). Thapsigargin was purchased from LC Laboratories (Läufelfingen, Switzerland), anti-Fas IgM (CH-11) from Medical and Biological Laboratories (Nagoya, Japan), Z-VAD-cmk from Enzyme Systems Products (Dublin, CA), BAPTA-AM from Molecular Probes (Eugene, OR), anti-α-fodrin monoclonal antibody from Affiniti (Nottingham, UK), and DEVD-AMC from Bachem (Bubendorf, Switzerland). The Apoptest-FITC kit from Nexins Research B.V. (Hoeven, The Netherlands), containing annexin-FITC, was used for assaying PS exposure. All other chemicals were from Sigma (St. Louis, MO).

2.2. Cell culture and preparation for experiments

Cells were cultured in RPMI-1640 with 10% heat-inactivated foetal calf serum and 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cultures were incubated at 37°C in humidified air with 5% carbon dioxide, and kept in logarithmic phase by routine passage every 2–3 days. Cells were centrifuged at $500 \times g$ for 5 min before being resuspended in fresh medium at 10^6/ml. In some experiments, cells were resuspended in media with 5 mM EGTA (pH 7.4). Other cells were incubated with 10 μM BAPTA-AM for 30 min before being centrifuged and resuspended in fresh medium. Unless otherwise stated, these procedures, plus addition of inhibitors, were all completed 20 min before stimulation. Stimulation was with either 100 nM thapsigargin, 12 ng/ml TNF with 1 μg/ml CHX, or 250 ng/ml anti-Fas antibody.

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Abbreviations: PS, phosphatidylserine; TNF, tumour necrosis factor; EGTA, ethylene glycol-bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; BAPTA-AM, acetoxymethyl ester of 1,2-bis(2-aminophenoxy)-ethane-*N,N′*-tetraacetic acid; FITC, fluorescein isothiocyanate; ICE, interleukin-1β converting enzyme; PARP, poly(ADP-ribose) polymerase; AMC, amino-4-methylcoumarin; cmk, chloromethylketone; CHX, cycloheximide; FSC, forward scatter; PMSF, phenylmethylsulfonyl fluoride

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2.3. PS exposure

PS exposure was measured by the binding of annexin V–FITC according to the protocol outlined by the manufacturers in the Apoptest-FITC kit. Cells were also stained with 100 μg/ml propidium iodide, before being analysed with a Becton Dickinson FACScan flow cytometer with a 15 mW 488 nm argon laser. Ten thousand cells were counted, and those taking up propidium iodide were gated out of subsequent analysis.

2.4. Cell blebbing and nuclear fragmentation

The number of cells with large membrane protrusions was counted by light microscopy. Samples were also centrifuged at $1000 \times g$ for 5 min and resuspended in 4% paraformaldehyde before being spread on slides coated with 3-aminopropyltriethoxysilane. The slides were rehydrated then stained with 10 μg/ml Hoechst 33342 for 10 min, rinsed in water, dried and sealed with Eukit and a coverslip. The fluorescent nuclei were viewed with a Leitz Diaplan fluorescent microscope. With both blebbing and nuclear fragmentation over 100 cells were assessed in two different fields of view.

2.5. Protease activation

The measurement of DEVD–AMC cleavage was modified from Nicholson et al. [26]. Lysates from 0.5 to 5×10^6 cells were added to microtitre plates along with 100 mM HEPES, 10% sucrose, 5 mM dithiothreitol, and 0.1% CHAPS at pH 7.25. The reaction was started by the addition of 33 μM DEVD–AMC, whose cleavage to release free AMC was monitored at an excitation of 355 nm and emission of 460 nm. Fluorescent units were converted to pmoles of AMC using a standard curve generated with free AMC.

2.6. Fodrin cleavage

Cells were pelleted and washed with ice-cold PBS (pH 7.1) containing 100 μM phenylmethylsulfonyl fluoride (PMSF) before being resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 100 μM PMSF) and boiled in a water bath for 5 min. Proteins were separated under reducing conditions at 120 V in 6% SDS–polyacrylamide gels and then Western blotted at 100 V for 2 h. Blots were blocked overnight in a high salt buffer (HSB) (50 mM Tris-base, 500 mM NaCl, 0.05% Tween-20) containing 3% bovine serum albumin, and then incubated for 1 h with anti-α-fodrin antibody diluted in HSB. After washing the blots 4 times for 5 min in HSB, they were incubated with a peroxidase-conjugated secondary antibody, and bound antibody was detected by enhanced chemiluminescence (Amersham).

3. Results

3.1. Induction of PS exposure with thapsigargin

Cells were labelled with annexin–FITC and those expressing PS on their outer surface were assessed by flow cytometry [24]. Necrotic cells, detected by their uptake of propidium iodide, were gated out before analysis was undertaken. Fig. 1 is an example of the appearance of a population of viable PS-positive U937 cells upon treatment with 100 nM thapsigargin. This exposure was both considerably slower and less than that reported with platelets. There was no detectable increase in PS exposure after 2 h (results not shown), but by 6 h 14% of the U937 and Jurkat cells expressed PS (Table 1). This corresponded to a 2.5-fold increase in the mean FITC fluorescence of U937 cells and a 2-fold increase in the Jurkat cells (Table 1).

During the flow cytometry analysis we also detected a small decrease in the forward scatter of the population (Table 1), indicative of the decrease in cell size that occurs during apoptosis. Two other characteristic morphological features of apoptosis, membrane blebbing and nuclear fragmentation, were also observed upon treatment of cells with thapsigargin, but only occurred in approximately 10% of the cells (Table 1).

One of the earliest changes observable in apoptotic cells is initiation of the interleukin-1β converting enzyme (ICE)-like proteolytic cascade [25]. A number of cysteine proteases with homology to ICE are activated and contribute to the cleavage of a variety of cellular substrates. One of these substrates, poly(ADP-ribose)polymerase (PARP), is cleaved at a specific Asp–Glu–Val–Asp (DEVD) sequence. This peptide has been linked to amino-4-methylcoumarin (AMC) to enable measurement of ICE-like protease activity in cell extracts, and the enzyme responsible for PARP cleavage was identified as CPP32/apopain [26,27]. After 6 h treatment with thapsigargin the level of DEVD–AMC cleavage had increased to 3 times the control level in U937 cells and by nearly 4 times in the Jurkat cells (Table 1). As with PS exposure, there was no increase in activity after 2 h (results not shown).

3.2. Mechanism of PS exposure upon thapsigargin treatment

EGTA was used to chelate extracellular Ca^{2+}. While EGTA does not affect the initial rise in intracellular Ca^{2+} triggered by thapsigargin, it does prevent the maintenance of these levels by abolishing the capacitative influx of extracellular Ca^{2+} [28]. Addition of 5 mM EGTA to the medium effectively inhibited PS exposure in both cell types, but did not inhibit DEVD–AMC cleavage in the cells (Table 2). This suggests that EGTA did not block stimulation by thapsigargin, although EGTA alone induced significant amounts of cleavage (Table 2). BAPTA-AM is commonly used to chelate intracellular Ca^{2+}, but it induced large amounts of apoptosis in both cell types over the 6 h period (results not shown).

In many systems inactivation of the ICE-like proteases can block apoptosis. The cell-permeable peptide inhibitor Val–Ala–Asp–chloromethylketone (VAD-cmk) blocked Jurkat cell PS exposure upon treatment with thapsigargin (Table

Table 1
Induction of apoptosis by thapsigargin, TNF/CHX or anti-Fas antibody

	PS exposure		FSC[c]	Membrane blebbing[d]	Nuclear frag.[e]	DEVD–AMC cleavage[f]
	% cells[a]	mean fluor.[b]				
U937 cells						
control	2.4 ± 0.2	32 ± 4	338 ± 5	0 ± 0	2.0 ± 0.2	12 ± 2
+thapsigargin	14 ± 2	80 ± 13	289 ± 5	8.5 ± 1.0	9.6 ± 0.4	37 ± 4
+TNF/CHX	49 ± 12	126 ± 44	179 ± 30	37 ± 7		102 ± 16
Jurkat cells						
control	3.7 ± 0.7	43 ± 9	318 ± 13	0 ± 0	1.6 ± 0.6	5.9 ± 1.3
+thapsigargin	14 ± 4	92 ± 22	291 ± 13	8.1 ± 1.6	9.2 ± 3.0	20 ± 4
+anti-Fas	43 ± 8	147 ± 29	227 ± 14	32 ± 3		53 ± 15

Cells were treated for 6 h with 100 nM thapsigargin, or for 2 h with either 12 ng/ml TNF and 1 μg/ml CHX, or 250 ng/ml anti-Fas antibody. Results are presented as the mean ± SE of at least three experiments. [a]Percentage of cells appearing as PS positive. [b]Mean annexin–FITC fluorescence of the viable population. [c]Mean forward scatter of the viable population. [d,e]Percentages of the population showing membrane blebbing or nuclear fragmentation. [f]pmol of DEVD–AMC cleaved per min per extract of 10^6 cells.

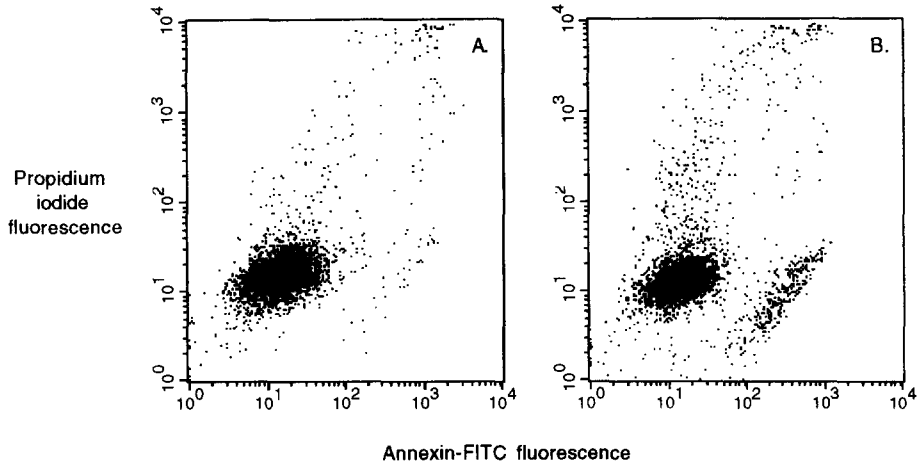

Fig. 1. Induction of PS exposure in U937 cells by thapsigargin. Cells were treated with 100 nM thapsigargin for 6 h before being washed and stained with annexin–FITC and propidium iodide. In each sample 10000 cells were analysed by flow cytometry. The fluorescence profiles of 25% of the (A) unstimulated and (B) thapsigargin-treated cells are shown from a representative experiment.

2), indicating that disruption of Ca^{2+} homeostasis led to PS exposure via activation of the ICE-like proteolytic cascade. VAD-cmk was ineffective in the U937 system even though it clearly inhibited protease activity (Table 2). However, VAD-cmk is known to be less effective in blocking TNF-mediated apoptosis in U937 cells ([9]; also see Table 3).

3.3. *Effect of EGTA on PS exposure during TNF- and Fas-mediated apoptosis*

Next we investigated two common models of receptor-mediated apoptosis: U937 cells stimulated with TNF and CHX, and Jurkat cells stimulated with anti-Fas antibody. In each model substantial apoptosis occurred within 2 h (Table).

Fig. 2 plots PS exposure versus forward scatter. A decrease in forward scatter is indicative of the decrease in size that occurs as a cell shrinks and fragments into smaller bodies during apotosis. TNF/CHX treatment resulted in a large proportion of U937 cells with both decreased forward scatter and increased PS exposure (Fig. 2B). Upon stimulating the cells in the presence of EGTA the cells still underwent a decrease in forward scatter, but this population now expressed considerably less PS (Fig. 2C). The same effect was observed in Jurkat cells (Fig. 2E–G). In contrast, VAD-cmk prevented both the size decrease and PS exposure in Jurkat cells (Fig. 2H).

Exposure is normally quantitated by classifying a cell as either PS-positive or PS-negative. We used the mean fluorescence of the population to better estimate the shift in annexin–FITC binding. Also, as EGTA increased the number of apoptotic cells (Table 3), and these cells have lower fluorescence because of their smaller surface area, we focussed on the apoptotic population. Analysis showed that EGTA inhibited PS exposure by 65% in apoptotic U937 cells (Fig. 2A). EGTA had a lesser effect in the Jurkat cells, but still decreased PS exposure by approximately 50% (Fig. 2B). EGTA did not inhibit any of the other apoptotic parameters, and in Jurkat cells in particular, actually increased the amount of apoptosis occurring (Table 3). So too did BAPTA-AM, but unlike EGTA, it did not inhibit PS exposure. Therefore the inhibition by EGTA was specific to PS exposure and was due to the removal of extracellular Ca^{2+} rather than disruption of intracellular levels.

Before PS exposure was measured the cells were washed in fresh PBS with 1 mM Ca^{2+}, then resuspended with the FITC–annexin in the presence of 1.8 mM Ca^{2+}; therefore EGTA was not present to interfere in the annexin binding assay. To confirm this we added EGTA at the end of a 2 h stimulation, but before the washing procedure, and saw no effect on PS exposure (Figs. 2D and Fig. 3). Also, identical inhibition was observed when we used Ca^{2+}-free RPMI with 0.5 mM EGTA (to chelate the Ca^{2+} in the 10% serum) (Fig. 3). The inhibition of PS exposure in the presence of 5 mM EGTA was reversible as resuspension of cells into normal medium resulted in a return to optimal PS exposure within 30 min (Fig. 3A).

The interaction of phospholipids with cytoskeletal proteins has been proposed to play a role in membrane asymmetry [2]. Cleavage of cytoskeletal α-fodrin occurs during apoptosis [29]. Western blotting shows that the major band of α-fodrin is at 240 kDa in control cells and that activation of apoptosis generates 150 and 120 kDa cleavage products (Fig. 4). Fodrin cleavage also occurred in the presence of EGTA (Fig. 4), indicating that even if cleavage occurred, it was insufficient for PS exposure.

Table 2
Effect of EGTA and VAD-cmk on thapsigargin-mediated apoptosis

	PS exposure	DEVD–AMC cleavage
	% of stimulated	
U937 cells and thaps	100	100
+EGTA	7 ± 20	145 ± 37
EGTA alone	−11 ± 18	42 ± 17
+VAD-cmk	80 ± 8	−26 ± 12
VAD-cmk alone	8 ± 2	−31 ± 12
Jurkat cells and thaps	100	100
+EGTA	29 ± 11	191 ± 32
EGTA alone	40 ± 25	89 ± 26
+VAD-cmk	−8 ± 0	−33 ± 0
VAD-cmk alone	−30 ± 0	−29 ± 0

Cells were incubated for 20 min in medium with 5 mM EGTA (pH 7.4) or with 10 μM VAD-cmk, before being treated with 100 nM thapsigargin for 6 h. Results (mean ± SE of 3 experiments) are presented as a percentage of thapsigargin-stimulated cells, after subtraction of the values for unstimulated cells.

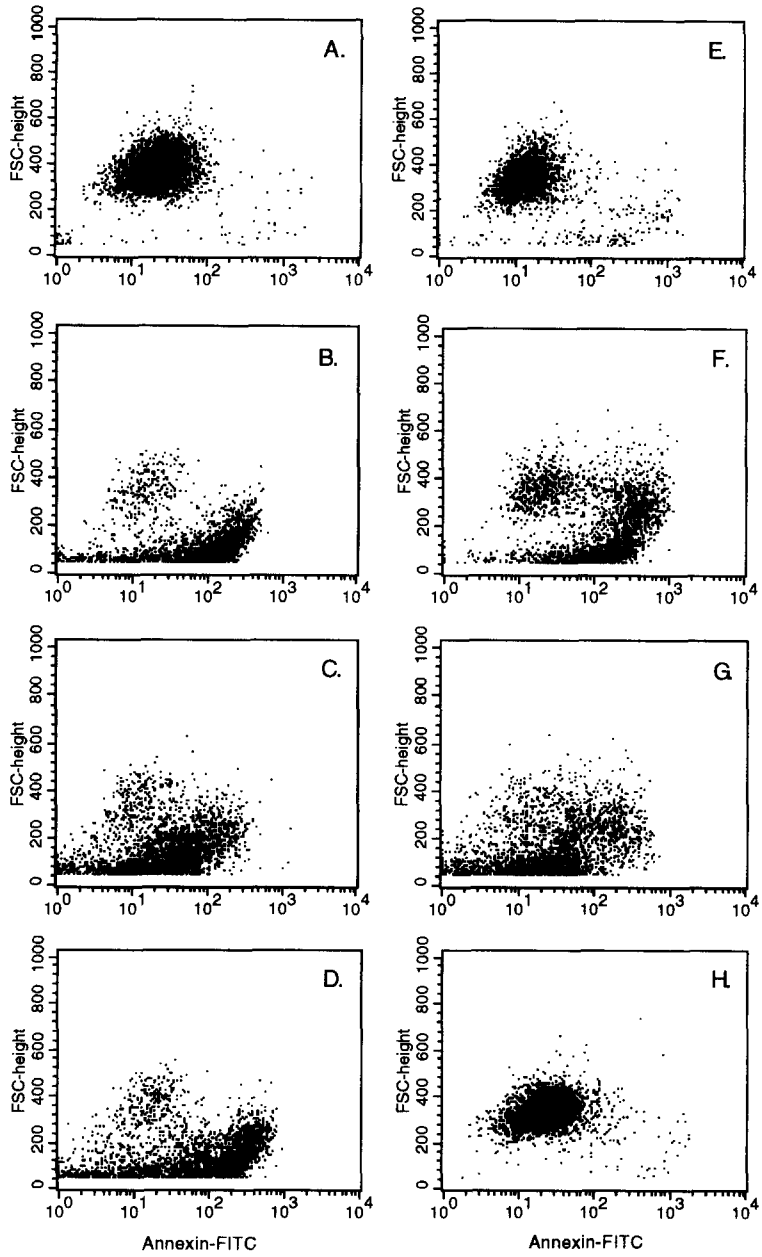

Fig. 2. PS exposure and forward scatter of TNF-treated U937 cells and anti-Fas-treated Jurkat cells. U937 cells (A–D) or Jurkat cells (E–H) were stimulated with TNF/CHX or anti-Fas, respectively, for 2 h before being stained with annexin-FITC and propidium iodide and analysed by flow cytometry. The forward scatter versus FITC fluorescence of 2500 propidium iodide-negative cells are shown from a representative experiment. A,E: Control cells. B,F: Stimulated cells. C,G: Stimulated in the presence of 5 mM EGTA. D: Addition of EGTA after the 2 h stimulation. H: Stimulated in the presence of 10 TM VAD-cmk.

4. Discussion

We have shown that triggering of apoptosis in U937 and Jurkat cells in the absence of extracellular Ca^{2+} leads to normal membrane blebbing, cell shrinkage, activation of the ICE-like proteolytic cascade, and fodrin cleavage. The apoptotic cells, however, have considerably less PS present on the outer surface of their membrane.

Platelets and red blood cells are the best studied models of PS exposure. In these systems an increase in intracellular Ca^{2+} can rapidly trigger PS exposure. While thapsigargin induced PS exposure in U937 and Jurkat cells, it was considerably slower and weaker, and was inhibited by the protease inhibitor VAD-cmk. This suggests that PS exposure was the result of slow activation of the apoptotic program, rather than a direct effect on the enzyme(s) responsible for PS exposure. While the mechanism for translocation of PS to the outer surface may be identical between these cell types, it is apparent that platelets have a different activation pathway. This may reflect the necessity of a rapid platelet response for blood coagulation.

The requirement for extracellular Ca^{2+} in PS exposure was clear. Our data suggest that the Ca^{2+} was involved at a late stage of the pathway, possibly by the enzyme(s) that mediates the transfer of PS to the outer leaflet of the membrane. EGTA inhibited only PS exposure, while it actually enhanced the other apoptotic parameters measured. Also, readdition of Ca^{2+} to apoptotic cells with impaired PS exposure resulted

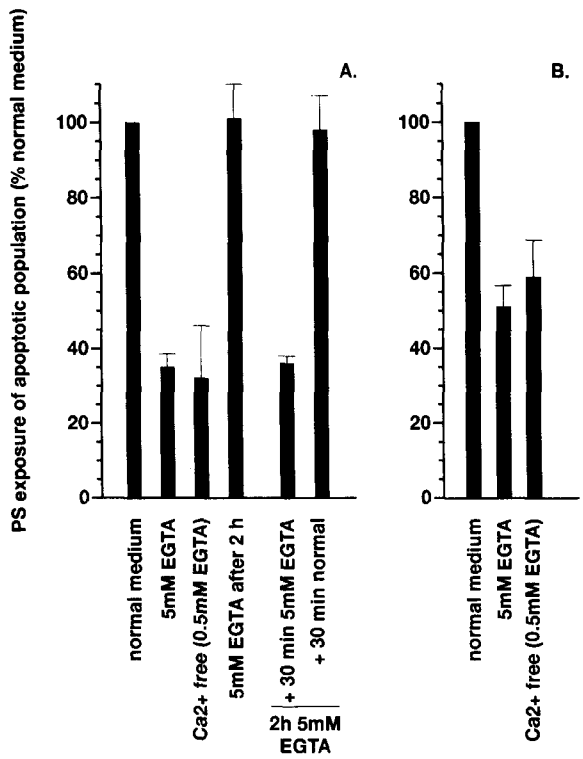

Fig. 3. Effect of EGTA on PS exposure in apoptotic U937 and Jurkat cells. (A) U937 or (B) Jurkat cells were stimulated with TNF/CHX or anti-Fas for 2 h before being stained with annexin–FITC and propidium iodide and analysed by flow cytometry. Results are calculated from the mean FITC fluorescence of the apoptotic population (those with decreased forward scatter), and expressed as a percentage of those stimulated in normal medium. Cells were tested in medium with 5 mM EGTA or Ca^{2+}-free medium with 0.5 mM EGTA. After the 2 h stimulation some cell samples were centrifuged and resuspended in normal or 5 mM EGTA media. The mean and SE of at least 3 experiments are shown.

in a rapid return to optimal exposure. This is what would be expected if all the necessary pathways had been activated by TNF or anti-Fas, but that the enzyme(s) involved in transferring the PS were lacking a crucial cofactor. The identity of these enzyme(s) is not yet clear. While it is known that Ca^{2+} can trigger activation in platelets and red blood cells, it is not yet known if Ca^{2+} is integral to activity when stimulated by an independent pathway. Confirmation of this will be dependent on further characterisation of the mechanics of PS exposure.

The cleavage of fodrin has been suggested for initiation of PS exposure [29]. We found that even if fodrin cleavage occurs it does not necessarily lead to PS exposure. However, both fodrin cleavage and flippase activity may be necessary for PS exposure. The critical experiment would therefore be activation of the flippase in the presence of intact fodrin.

EGTA was used to chelate extracellular Ca^{2+}, but it also lowers intracellular levels by preventing the capacitative filling of depleted stores [30]. As such, it has been used to study the role of Ca^{2+} in triggering the apoptotic program. Some see an inhibition of apoptosis with Ca^{2+} chelators [21] and others an enhancement [22,31]. Results vary dramatically, depending on the cell type investigated. For example, our results with the Jurkat cells support those of Weis et al. [32] showing that Fas-mediated apoptosis is not affected by removal of Ca^{2+}, but Rovere et al. [33] showed that BAPTA inhibited apoptosis, including PS exposure, in a human γδ T-cell clone.

A decrease in intracellular Ca^{2+} by EGTA could have been responsible for the impairment of PS exposure that we detected. However, as elevating intracellular Ca^{2+} with thapsigargin was an inefficient trigger of PS exposure in U937 and Jurkat cells, and the intracellular chelator BAPTA-AM did not prevent TNF- and anti-Fas-mediated PS exposure, we believe that extracellular Ca^{2+} is most likely to be involved. The small amount of PS exposure that was stimulated with thapsigargin was also inhibited with EGTA, supporting the TNF and anti-Fas results. In this system EGTA did not appear to block the thapsigargin signal, as ICE-like protease activation still occurred. As the thapsigargin-mediated increase in intracellular Ca^{2+} is not sustained in the presence

Table 3
Effect of Ca^{2+} chelators and VAD-cmk on TNF and Fas-mediated apoptosis

	PS exposure	FSC	Membrane blebbing	DEVD cleavage
	% of stimulated			
U937 and TNF/CHX	100	100	100	100
+EGTA	*	109 ± 15	105 ± 10	130 ± 25
EGTA alone	−1 ± 10	2 ± 4	1 ± 1	3 ± 2
+BAPTA-AM	138 ± 32	82 ± 7	113 ± 25	149 ± 22
BAPTA-AM alone	20 ± 9	15 ± 6	7 ± 6	14 ± 9
+VAD-cmk	87 ± 22	69 ± 13	34 ± 5	−11 ± 12
VAD-cmk alone	4 ± 0	6 ± 0	0 ± 6	−37 ± 34
Jurkat and Fas	100	100	100	100
+EGTA	*	174 ± 30	188 ± 12	174 ± 29
EGTA alone	−19 ± 6	9 ± 5	2 ± 2	7 ± 3
+BAPTA-AM	256 ± 34	152 ± 43	259 ± 30	266 ± 56
BAPTA-AM alone	24 ± 11	24 ± 17	12 ± 8	36 ± 9
+VAD-cmk	11 ± 8	14 ± 8	7 ± 7	−11 ± 5
VAD-cmk alone	−1 ± 12	4 ± 0	0 ± 0	−12 ± 9

Cells were incubated for 20 min in medium with 5 mM EGTA (pH 7.4), with 10 μM VAD-cmk, or for 30 min with 10 μM BAPTA-AM before being centrifuged and resuspended in fresh media. U937 cells were then treated for 2 h with 12 ng/ml TNF and 1 μg/ml CHX, and Jurkat cells were treated for 2 h with 250 ng/ml anti-Fas antibody. Results (mean ± SE of at least 3 experiments) are presented as a percentage of stimulated cells, after subtraction of the values for unstimulated cells. *The effect of EGTA on PS exposure is presented in Fig. 3.

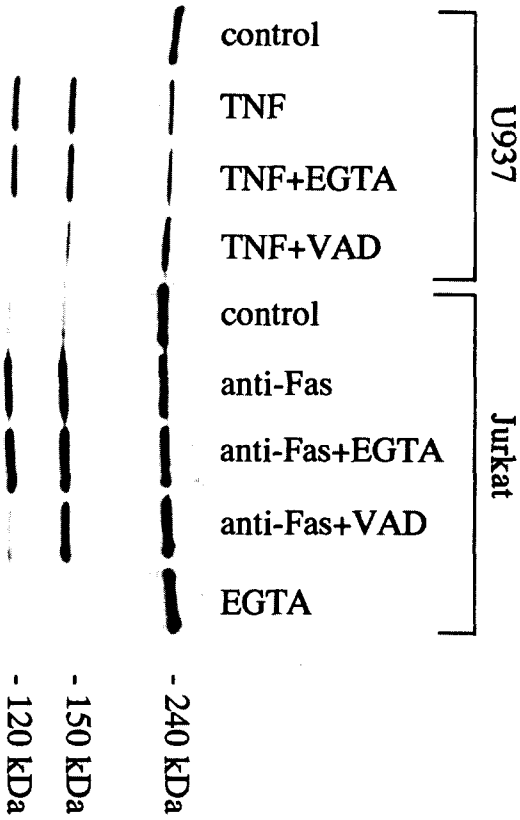

Fig. 4. Effect of EGTA on fodrin cleavage in apoptotic Jurkat and U937 cells. U937 cells were treated with TNF (20 ng/ml) and CHX (1 μg/ml), and Jurkat cells were treated with anti-Fas (250 ng/ml) for 2 h. Some cell samples were pre-incubated with VAD-cmk (10 μM) or EGTA (5 mM) for 20 min. The incubation was stopped by addition of cold PBS and the α-fodrin content of lysed cells was analysed by Western blotting. The blot is from a representative experiment.

of EGTA [28], it is possible that apoptosis was triggered by a slow depletion of endoplasmic reticulum Ca^{2+} stores. The observed activation of apoptosis by EGTA or BAPTA-AM may also have occurred by the same mechanism.

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