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Increased cell surface exposure of phosphatidylserine on propidium iodide negative thymocytes undergoing death by necrosis

Paul Waring*,[1], Damaris Lambert[2], Allan Sjaarda[1], Alanna Hurne[1] and Joanne Beaver[2]

[1] Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, PO Box 334, Canberra, Australia
[2] Research Centre for Human and Biomedical Sciences, Faculty of Applied Science, University of Canberra, PO Box 1, Belconnen, Canberra, Australia
* corresponding author: Division of Immunology and Cell Biology, John Curtin School of Medical Research, Australian National University, PO Box 334, Canberra, Australia; e-mail: Paul.Waring@anu.edu.au; fax: 0262492595

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Abstract

Phosphatidylserine (PS) exposure on propidium iodide negative cells using FITC labelled annexin-V has been used to quantify apoptosis *in vitro* and *in vivo*. Detection of PS within cells undergoing necrosis is also possible if labelled annexin-V specific for PS enters the cell following early membrane damage. Necrotic or late apoptotic cells can be excluded from flow cytometric analysis using propidium iodide which enters and stains cells with compromised membrane integrity. Here we show that thymocytes undergoing death exclusively by necrosis show early exposure of PS prior to loss of membrane integrity. This early exposure of PS occurs in cells treated with agents which both raise intracellular calcium levels and are also capable of interacting with protein thiol groups. We also demonstrate that PS exposure in thymocytes induced to undergo apoptosis by three different agents does not correlate with calcium rises but correlates with and precedes DNA fragmentation.

Keywords: phosphatidylserine; apoptosis; necrosis; calcium; thiols

Abbreviations: c-AMP, cyclic AMP; 2,2′ DDP, 4,4′ DDP, 2,2′ dithiodipyridine, 4,4′ dithiodipyridine; DTNB, 5,5′ dithiobis(2-nitrobenzoic acid); NEM, N-ethylmaleimide; PI, propidium iodide; PS, phosphatidylserine; PS^+PI^+, phosphatidylserine positive and propidium iodide positive cell populations; PS^+PI^-, phosphatidylserine positive and propidium iodide negative cell populations

Introduction

The general observation of the exposure of phosphatidylserine (PS) on the surface of cells undergoing apoptosis has led to the measurement of PS exposure, usually by estimation of labelled annexin-V binding, as diagnostic of apoptosis.[1–4] Exposure of PS is thought to trigger removal of cells *in vivo* by resident phagocytes[5] and has been demonstrated in a wide variety of *in vitro* and *in vivo* models of apoptosis.[6–10] Cells in culture are particularly amenable to the use of this assay using flow cytometry. In this assay it is usual to measure propidium iodide (PI) uptake in cells and exclude those PI positive cells from analysis since these will include necrotic cells[6] although PI positive cells will also include apoptotic cells undergoing secondary necrosis. Necrotic cells with leaky plasma membranes may show an apparent increase in PS as assessed by annexin V binding presumably due to increased access of the probe into the intracellular milieu since PS is normally located on the inner leaflet of the plasma membrane facing the cytosol.

The detailed pathways controlling exposure of PS during apoptosis are not known but the process is controlled by modulation of the enzymes such as aminophospholipid transferases present in the cell which retain PS on the inner leaflet of the plasma membrane.[11,12] Caspases have been suggested to play a direct role in exposure of PS[7,13,14] but this has been disputed.[15] Rises in intracellular calcium in platelets has been shown to trigger rapid exposure of PS in these cells[16] and it has been suggested that calcium rises in apoptotic cells may also be responsible for triggering PS exposure. Intracellular calcium rises have been shown to be associated with the onset of apoptosis in some cases,[17] but they are not universally associated with the early stages of the process.[18] Calcium influx through the membrane may be a late event in apoptosis as the cells undergo secondary necrosis due to influx of extracellular calcium modulated by increases in reactive oxygen species.[19]

We initially set out to show that PS exposure correlated strictly with DNA fragmentation not intracellular calcium rises in thymocytes using three disparate agents already shown to induce apoptosis; dexamethasone, gliotoxin and thapsigargin. The results for gliotoxin, a fungal metabolite with a strained disulphide bridge, led us to examine the effects of the thiol specific agents 2,2′ and 4,4′ dithiodipyridine (2,2′DDP, 4,4′DDP) and N-ethylmaleimide (NEM) on PS exposure, calcium rises and apoptosis in thymocytes. The results showed that PS exposure as assessed by annexin V binding can occur in necrotic thymocytes prior to propidium iodide uptake and indicated that caution should be used when assessing apoptosis by PS exposure alone.

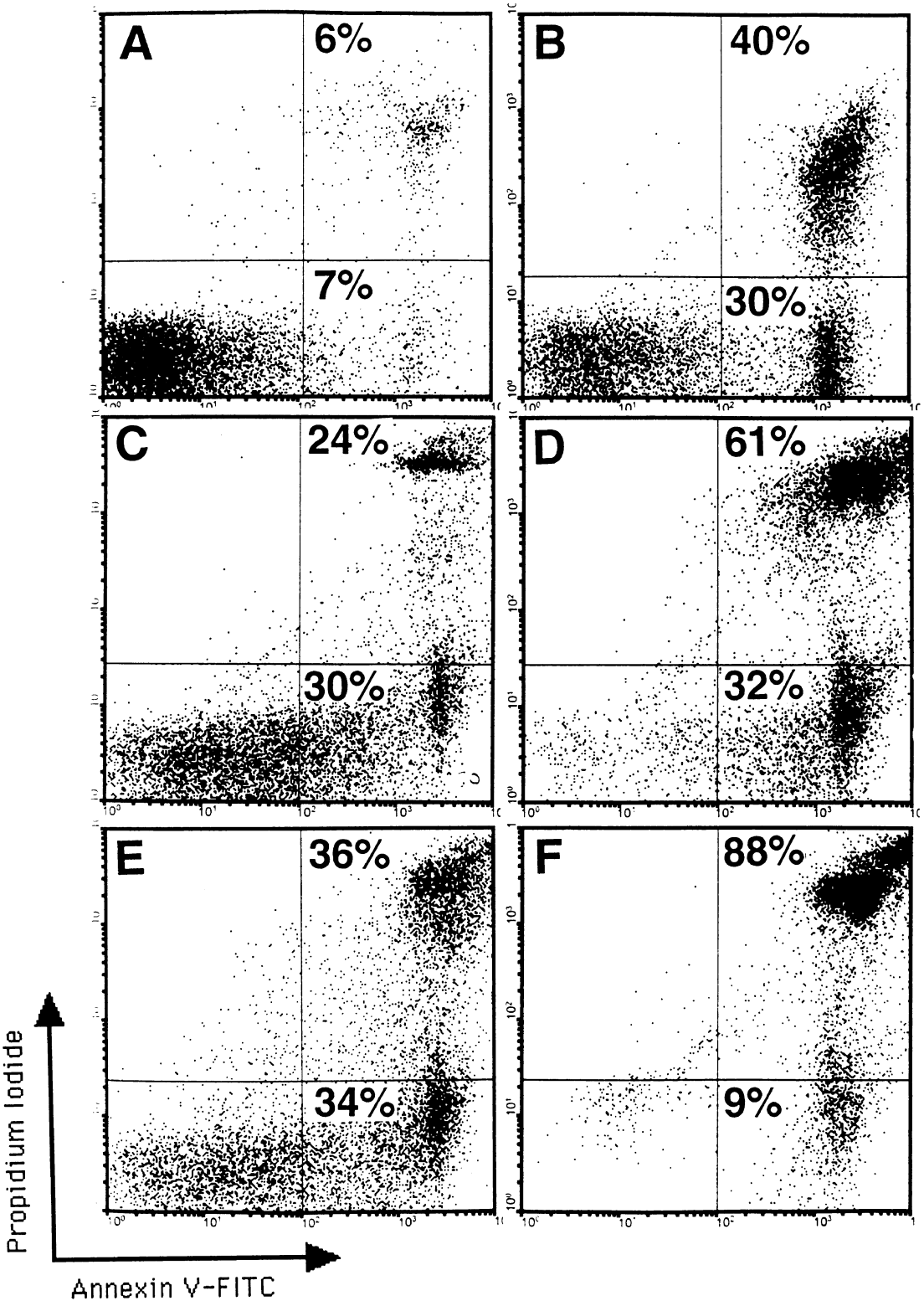

Figure 1 Representative examples of phosphatidylserine exposure on thymocytes incubated with dexamethasone and gliotoxin. (**A**) Untreated 6 h. (**B**) 1 μM dexamethasone 6 h. (**C**) 3 μM gliotoxin 1 h. (**D**) 100 μM gliotoxin 1 h. (**E**) 3 μM gliotoxin 3 h. (**F**) 100 μM gliotoxin 3 h

Results

PS exposure induced by dexamethasone, gliotoxin and thapsigargin correlates with DNA fragmentation and not early calcium rises

In order to examine the temporal relationship between PS exposure and DNA fragmentation, we chose three disparate agents that are known to induce apoptosis in thymocytes by different routes. The classical features of apoptosis in thymocytes induced by dexamethasone is dependent on macromolecular synthesis[20] following binding of the hormone analogue to the glucocorticosteroid receptor and transport to the nucleus. Gliotoxin induces rapid c-AMP rises in thymocytes and phosphorylation of histone H3 which we have shown is associated with the induction of apoptosis.[21] We have earlier shown that thymocytes from 10 day-old Balb/c mice undergoing apoptosis induced by dexamethasone or gliotoxin display no early intracellular calcium rises within 80 min.[18] In contrast, thapsigargin induces rapid calcium rises in thymocytes within minutes through specific inhibition of the ATP-dependent Ca^{2+} endoplasmic reticulum pump. Thapsigargin induced apoptosis in thymocytes is preceded by mitochondrial damage[22] and thus may be triggered by release of apoptogenic factors such as cytochrome c.[23] We have shown that gliotoxin at 1–3 μM and thapsigargin at 10 nM induce exclusively apoptosis, as distinct from necrosis, in thymocytes from 10 day-old Balb/c mice[18] and dexamethasone is a well described inducer of apoptosis in thymocytes.[20]

Thymocytes treated with all three agents displayed increased exposure of PS. Double staining with propidium iodide and annexin-V-FITC showed the typical population of high PS low PI staining cells (PS^+/PI^-) characteristic of apoptotic cells. Figure 1B shows a typical profile of thymocytes treated with 1 μM dexamethasone for 6 h compared to untreated cells in Figure 1A. Figure 1C and E shows result for gliotoxin at 3 μM at 1 and 3 h. Cells typically move through the lower right quadrant to the upper right quadrant with time to become high PS high PI staining cells (PS^+/PI^+). This was shown in a kinetic study in Figure 2 for cells treated with 1 μM dexamethasone. For dexamethasone treated cells the PS^+/PI^- population peaks at about 25% at 2–2.5 h. DNA fragmentation is a late event as expected and correlates with the number of cells in the PS^+/PI^+ region. The onset of DNA fragmentation occurred at a later time in thapsigargin treated thymocytes consistent with earlier work[24] as shown in Figure 2B. The profile for gliotoxin at 3 μM (not shown) was very similar to that of dexamethasone. All three agents resulted in an increased population of cells with DNA fragmentation which was preceded by an increased population of cells with increased total PS (i.e. the sum of the cells in the PS^+PI^- and PS^+PS^+ regions). Sorting experiments discussed below showed that both the high and low PI stained cells treated with the above agents and with increased PS exposure showed DNA fragmentation but the majority of cells with fragmented DNA were in the PS^+PI^+ region.

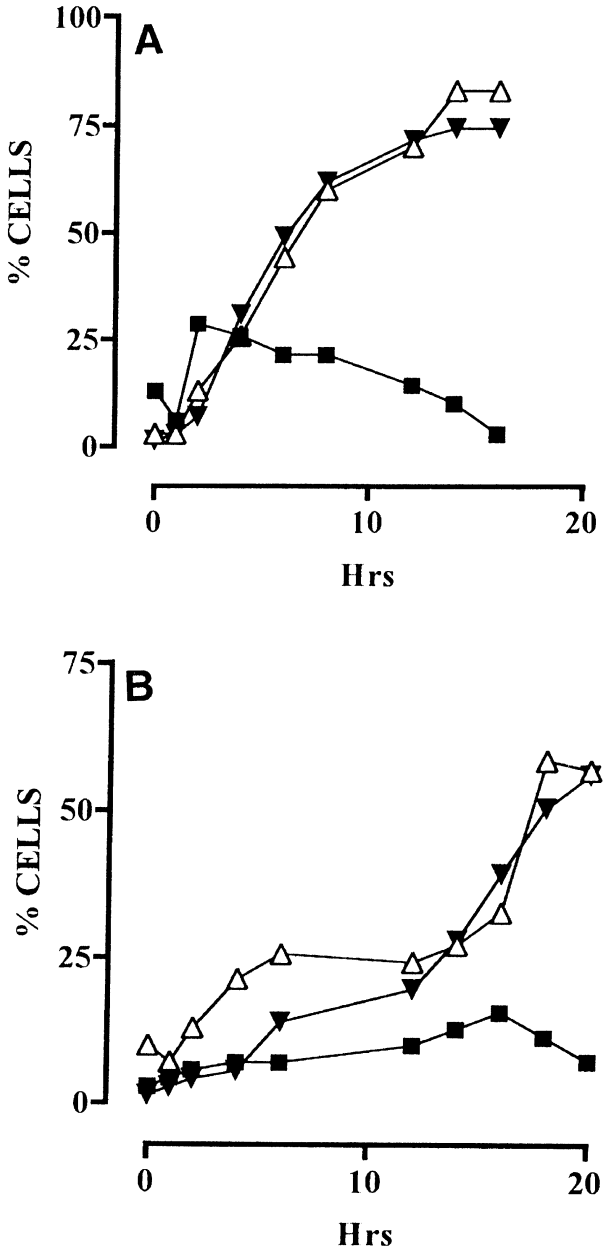

Figure 2 Kinetics of appearance of PS^+PI^-, PS^+PI^+ and DNA fragmentation in thymocytes treated with 1 μM dexamethasone (**A**) or 10 nM thapsigargin (**B**). Closed square: proportion of cells PS^+PI^-. Open triangle: Proportion of cells PS^+PI^+. Closed triangle: proportion of cells with DNA fragmentation. Each data point is the mean of three determinations. Error bars are left out for clarity. S.D. < 15%

PS exposure and the nature of cell death in thymocytes treated with sulphydryl agents

Like many toxins, gliotoxin induces apoptosis within a range of concentrations which we have previously defined at 0.1–10 μM. Above 10 μM cell death induced by gliotoxin switches to necrosis and 100 μM gliotoxin leads exclusively to necrosis.[18] Interestingly we observed

that treatment of thymocytes with 100 μM gliotoxin for 1–2 h resulted in the appearance of a population of cells with increased PS exposure but which were PI negative. Thus in Figure 1D it can be seen that 32% of thymocytes after treatment with 100 μM gliotoxin for 1 h were in the PS^+PI^- compared to 30% of cells treated

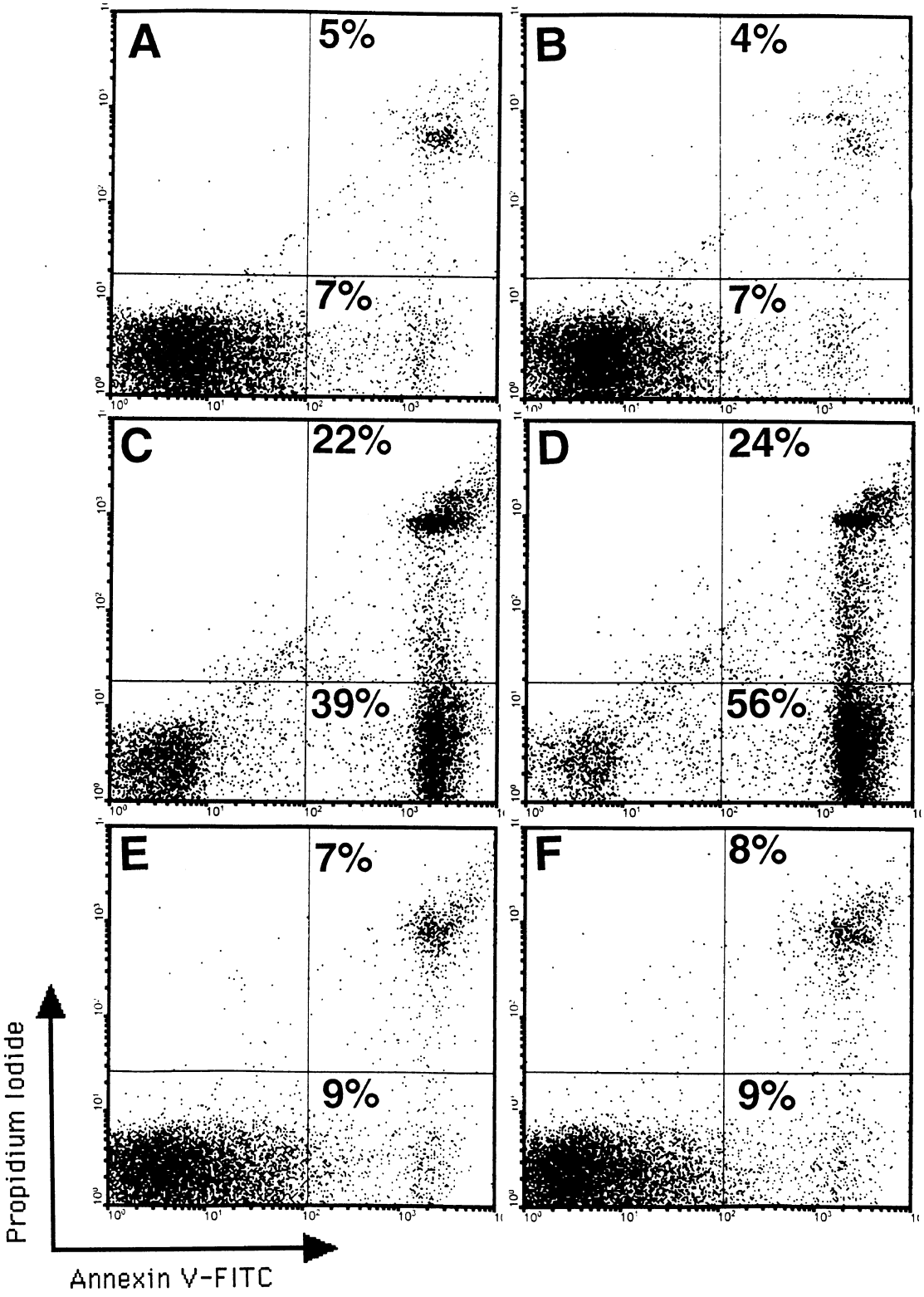

Figure 3 Representative examples of phosphatidylserine exposure on thymocytes incubated with NEM. (**A**) Untreated 6 h. (**B**) 3 μM NEM 6 h. (**C**) 10 μM NEM 6 h. (**D**) 40 μM NEM 6 h. (**E**) 100 μM DTNB 6 h. (**F**) 10 μM DTNB 6 h

with 1 μM dexamethasone at 6 h. Higher concentrations such as 300 μM gliotoxin did not result in the appearance of this population and most cells were found in the upper right quadrant even after 1 h (not shown). By 3 h cells treated with 100 μM gliotoxin had entered the PS^+PI^+ region.

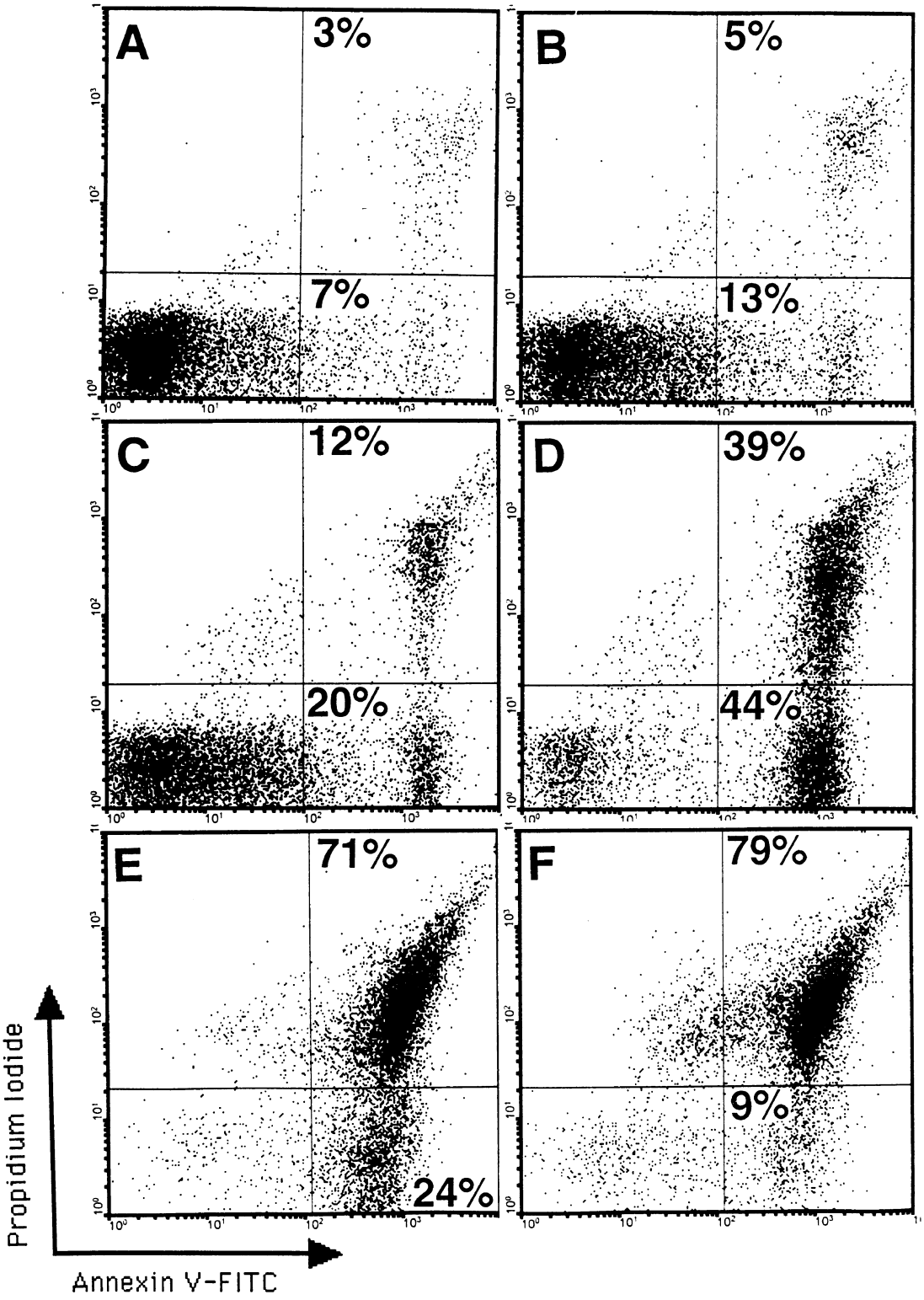

Figure 4 Representative examples of phosphatidylserine exposure on thymocytes incubated with 4,4′ DDP. (**A**) Untreated 3 h. (**B**) 3 μM 4,4′ DDP 3 h. (**C**) 10 μM 4,4′ DDP 3 h. (**D**) 40 μM 4,4′ DDP 3 h. (**E**) 40 μM 4,4′ DDP 3 h in calcium free (<100 μM) media. (**F**) 40 μM 4,4′ DDP 3 h in calcium free (<100 μM) media and 500 μM EDTA

Gliotoxin belongs to the epipolythiodioxopiperazine (ETP) class of fungal metabolites[25] and these compounds are characterised by the presence of a bridged polysulphide piperazine ring, typically a disulphide bridge in the case of gliotoxin. Because this disulphide has been shown to react with free thiol groups on proteins,[26–28] we examined the effects of a series of thiol specific agents on PS exposure in thymocytes. The agents 2,2′ dithiodipyridine (2,2′ DDP), 4,4′ dithiodipyridine (4,4′ DDP), 5,5′ dithiobis(2-nitrobenzoic acid) (DTNB), N-ethylmaleimide (NEM) are all commonly used to react with accessible protein thiols. NEM alkylates reactive thiols while the other agents form mixed disulphides with the reactive thiol.

Figures 3 and 4 show representative effects of these agents on PS exposure in thymocytes. There is a concentration dependent increase in a population of cells in the PS^+PI^- region caused by NEM after 6 h incubation with the maximum exposure of PS without PI uptake occurring at 40 μM at 6 h as shown in Figure 3B–D. In addition, Figure 3 also shows that DTNB up to 100 μM caused no PS exposure. Figure 4 shows that 4,4′ DDP also resulted in a significant population of cells entering the PS^+PI^- region by 3 h with maximal effect at 40 μM as Figure 4D shows. Extracellular calcium has been shown to be required for PS exposure in apoptotic cells.[29] Removal of extracellular calcium by use of calcium free media or chelation with EGTA in the experiments described here resulted in cells directly entering the PS^+PI^+ region as shown in Figure 4E and F. Figure 5 shows the relative effect of 3 μM 2,2′ DDP on PS exposure compared to dexamethasone at 1 μM. The effect of 3 μM 2,2′ DDP was much more rapid than that caused by dexamethasone although the number of cells in the PS^+PI^- region at 6 h were similar. The result in Figure 6 showed that the major effect of NEM and 4,4′ DDP on PS exposure occurs only at concentrations exceeding 10 μM.

We also examined the nature of the death in cells treated with these sulphydryl agents. We could detect no sign of DNA fragmentation in thymocytes treated with 2,2′ DDP, 4,4′ DDP and NEM up to 24 h post treatment using a range of concentrations from 10 nM to 100 μM (not all data shown). Figure 7 shows representative data for these experiments. The proportion of cells undergoing DNA fragmentation was assessed using the propidium iodide staining of ethanol fixed cells. Gliotoxin at 3 μM and dexamethasone at 1 μM resulted in a significant proportion of cells in the subdiploid region at 6 h. Incubation with 4,4′ DDP at 10 μM and 40 μM and 2,2′ DDP at 3 μM resulted in no increase in the subdiploid population of cells.

At 24 h, 1 μM dexamethasone resulted in 70% of the cells with fragmented DNA as shown in Figure 8B. Gliotoxin gave similar results to 1 μM dexamethasone at 24 h (data not shown). Again Figure 8 shows that no DNA fragmentation was seen at 24 h in thymocytes treated with 3 μM 2,2′ DDP or NEM at 10, 40 or 100 μM. In fact in all experiments the background level of DNA fragmentation in cells treated with NEM, 4,4′ and 2,2′ DDP at any concentration above 3 μM was less than that seen in untreated cells. This apparent reduction in DNA fragmentation has been consistently observed in this laboratory to be characteristic of agents which induce necrotic cell death in thymocytes.

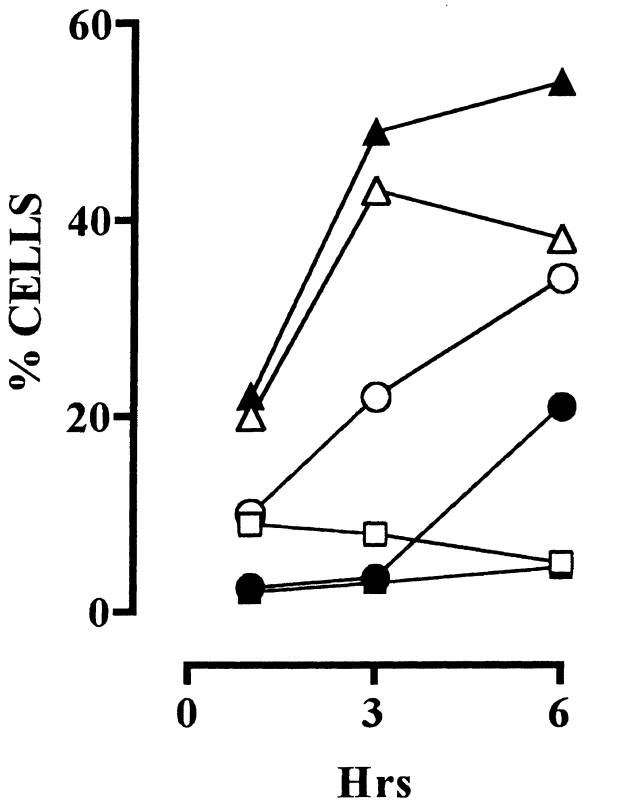

Figure 5 Kinetics of appearance of PS^+PI^- thymocytes treated with dexamethasone and 2,2′ DDP. Open squares: untreated cells, PS^+PI^-; closed squares: untreated cells PS^+PI^+; ○: 1 μM dexamethasone, PS^+PI^-; ●: 1 μM dexamethasone, PS^+PI^+; △: 3 μM 2,2′ DDP, PS^+PI^-; ▲: 3 μM 2,2′ DDP, PS^+PI^+. Each data point is the average of two values. Typical of two separate experiments

In order to confirm that PS^+PI^- cells had not undergone any DNA fragmentation, we sorted both the PS^+PI^- and the PS^+PI^+ populations and examined them for DNA fragmentation. Figure 9 shows that after sorting thymocytes treated with 1 μM dexamethasone, the PS^+PI^- population showed about 22% of cells with DNA fragmentation while the PS^+PI^+ population showed about 65% of cells with fragmentation. This result is consistent with PS exposure preceding DNA fragmentation and with Figure 2. We have also found very similar results for thymocytes treated with 3 μM gliotoxin (data not shown). PS^+PI^- and PS^+PI^+ sorted cells from 4,4′ DDP treated thymocytes on the other hand showed no such fragmentation as shown in Figure 9. In addition, when PS^+PI^- sorted cells from 4,4′ DDP treated thymocytes at 6 h were returned to culture for 18 h, DNA fragmentation could not be detected (data not shown). Figure 10 shows that no internucleosomal DNA fragmentation as assessed by agarose gel electrophoresis was observed in thymocytes treated with either 100 μM gliotoxin, 10 μM NEM or 40 μM 4,4′ DDP for 6 h. The typical apoptotic DNA laddering is

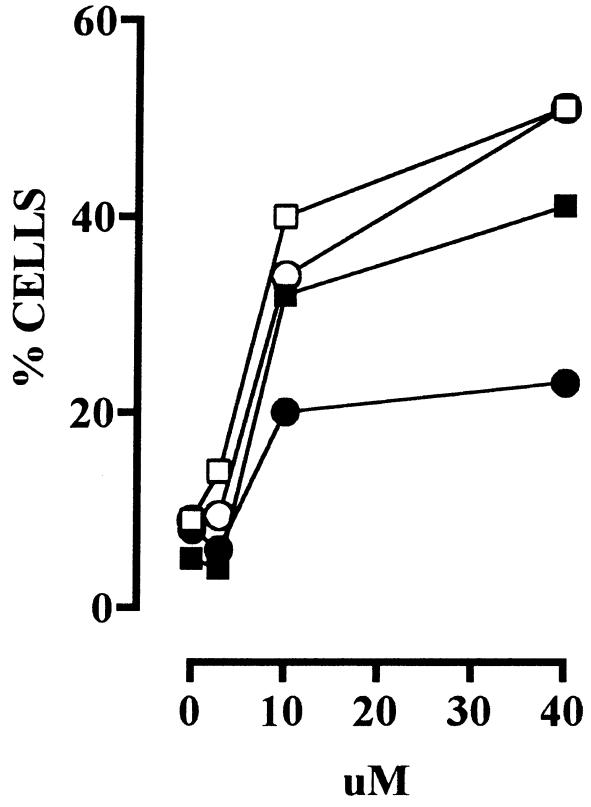

Figure 6 Concentration dependence of PS exposure on thymocytes treated with 4,4′ DDP and NEM 6 h. □: 4,4′ DDP, PS^+PI^-; ■: 4,4′ DDP, PS^+PI^-; ○: NEM, PS^+PI^-; ●: NEM, PS^+PI^+. Each data point is the average of two values. Typical of two separate experiments

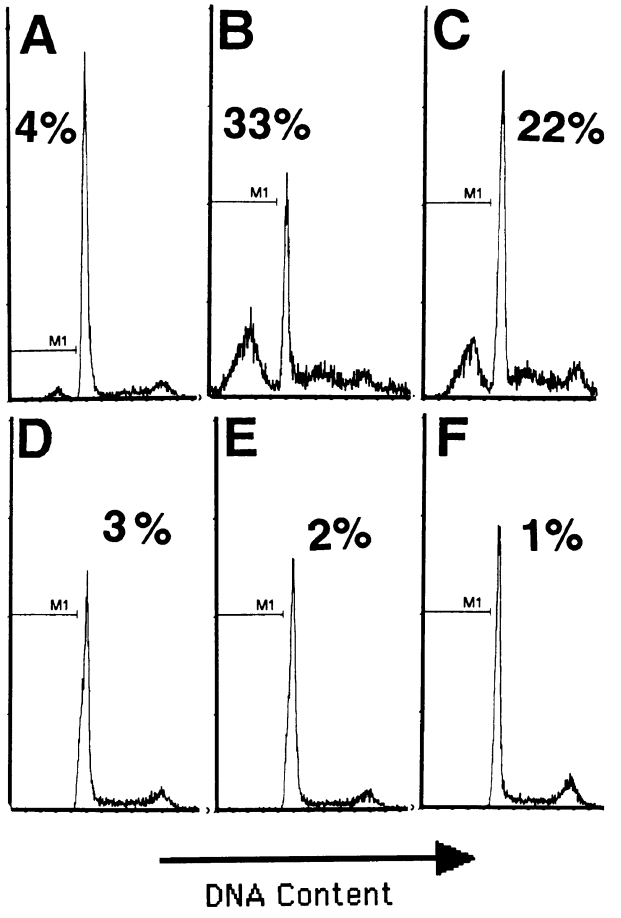

Figure 7 No DNA fragmentation induced in thymocytes by DDPs at 6 h. (**A**) Untreated. (**B**) 3 μM gliotoxin. (**C**) 1 μM dexamethasone. (**D**) 10 μM 4,4′ DDP. (**E**) 40 μM 4,4′ DDP. (**F**) 3 μM 2,2′ DDP

seen in cells treated with 1 μM dexamethasone or 3 μM gliotoxin for the same time.

Morphology of treated thymocytes

Using electron microscopy, we also carried out an extensive morphological examination of cells treated with 2,2′ DDP, 4,4′ DDP and NEM at the concentrations and times which resulted in the appearance of the PS^+PI^- cell population. At no time did we detect any of the features of apoptosis in these cells as Figure 11 shows. Cell death induced by 2,2′ DDP, 4,4′ DDP and NEM at all concentrations studied had the characteristics of necrotic cell death with swollen organelles, disrupted plasma membrane, loss of internal structure and no indication of condensed chromatin. These features can be clearly seen in Figure 11G–L. Extensive examination of cells treated with 2,2′ DDP, 4,4′ DDP and NEM over a wide range of concentrations and time points failed to detect any cells with apoptotic morphology. Cell death induced by dexamethasone at 1 μM and gliotoxin at 3 μM showed all the expected features of condensed chromatin with intact plasma membranes at the 6 h time point and secondary necrotic cells at the 24 h time point typical of apoptosis induced by these agents in culture as Figure 11C–F illustrates.

Calcium rises in thymocytes induced by sulphydryl specific agents

We have already reported that gliotoxin at concentrations above 50 μM induced calcium rises in thymocytes.[18] We therefore examined whether the sulphydryl agents used in this study also caused rises in intracellular calcium. These results are summarised in Figure 12 which also includes data for dexamethasone, gliotoxin and thapsigargin. Thapsigargin at 10 nM shows a rapid and sustained rise in intracellular calcium. The initial rise, then fall, may be due to the action of plasma membrane pumps as previously reported.[24] Neither gliotoxin at 3 μM, DTNB at 100 μM or dexamethasone at 1 μM caused any calcium rise over 80 min. In addition, dexamethasone treated thymocytes showed no change in intracellular calcium up to 2.25 h as shown in Figure 12, top panel, inset. It is clear that, with the exception of thapsigargin, those agents studied here which cause a rapid rise in calcium in thymocytes result in both rapid expression of PS^+PI^- cells and necrosis. This is also true for gliotoxin at 100 μM. Significantly DTNB does not cause early calcium rises in treated thymocytes.

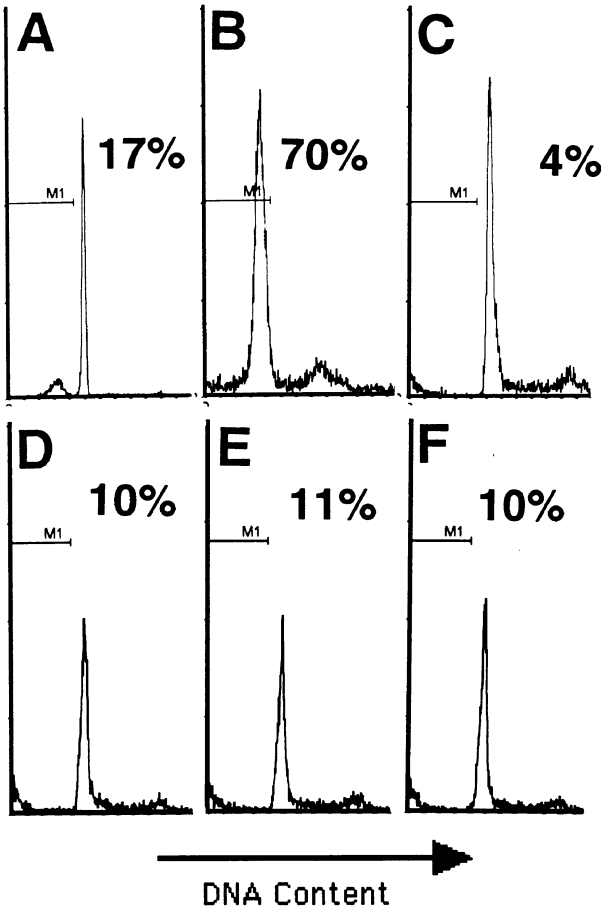

Figure 8 No DNA fragmentation induced in thymocytes by 2,2′ DDP or NEM at 24 h. (**A**) Untreated. (**B**) 1 μM dexamethasone. (**C**) 3 μM 2,2′ DDP; (**D**) 10 μM NEM. (**E**) 40 μM NEM. (**F**) 100 μM NEM

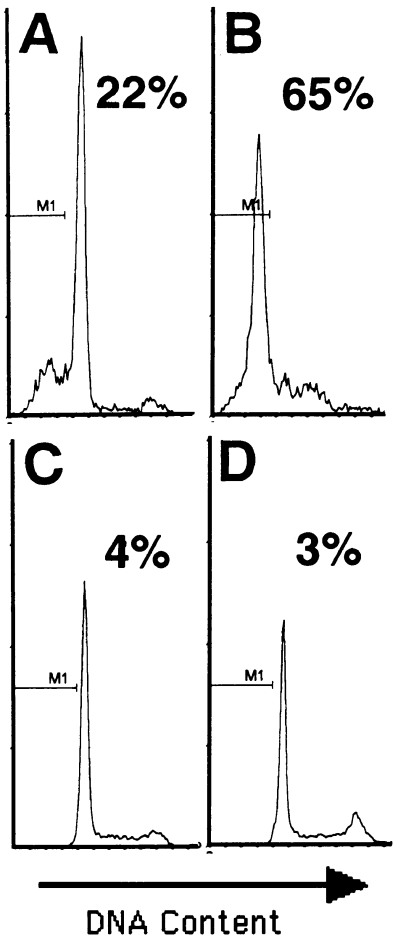

Figure 9 DNA fragmentation in sorted cells from PS^+PI^- and PS^+PI^+ populations. (**A**) 1 μM dexamethasone 6 h, PS^+PI^- population. (**B**) 1 μM dexamethasone 6 h, PS^+PI^+ population. (**C**) 40 μM 4,4′ DDP 3 h, PS^+PI^- population. (**D**) 40 μM 4,4′ DDP 3 h, PS^+PI^+ population

Increased intracellular free calcium inhibits PS exposure by dexamethasone

We have previously reported that thapsigargin inhibits the early DNA fragmentation induced by dexamethasone[30] and that thymocytes treated with thapsigargin and dexamethasone proceed to apoptosis with the same (delayed) kinetics of thymocytes treated with thapsigargin alone as in Figure 2B. We therefore examined whether thapsigargin would inhibit PS exposure caused by dexamethasone at 6 h. This is the optimal time for maximal PS^+PI^- exposure in thymocytes induced by dexamethasone treatment alone. Figure 13 shows that thapsigargin does indeed delay PS exposure induced by dexamethasone. In this experiment thapsigargin alone results in about 15% of cells entering the PS^+PI^- region compared to 31% for dexamethasone at 1 μM. Co-incubation with thapsigargin at 25 nM and dexamethasone at 1 μM results in 15% of PS^+PI^- cells–the same as thapsigargin. This is entirely consistent with earlier reports that thapsigargin inhibits DNA fragmentation caused by dexamethasone.

Thapsigargin mobilizes a different calcium pool from 4,4′ dithiodipyridine

Both thapsigargin and the thiol specific agents such as 2,2′ and 4,4′ DDP both cause significant calcium rises in thymocytes yet PS exposure induced by the former occurs over a much longer time period than the latter agents and correlates with DNA fragmentation. The thiol specific agents cause a very rapid appearance of PS on the cell surface as assessed by annexin V binding. We have evidence that gliotoxin and its analogs and 2,2′ and 4,4′ DDP interacts with a membrane associated thiol group to allow direct entry of calcium into the cell. This occurs before overt damage to the plasma membrane (Hurne, Waring *et al*, in preparation). We used the calcium sensitive dye FIP-18, which when loaded into cells should report near membrane calcium rises, to assess if there are qualitative differences between the calcium rises induced by thapsigargin and 4,4′ DDP. Figure 14 shows a comparison of calcium rises in thymocytes reported by INDO-1 and FIP-18 induced by either thapsigargin or 4,4′

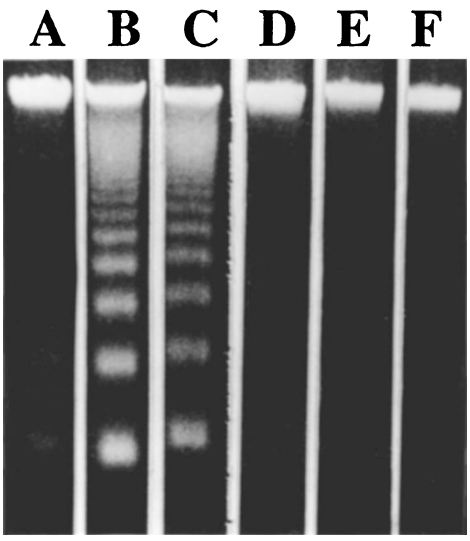

Figure 10 DNA laddering was induced in thymocytes by dexamethasone and 3 μM gliotoxin but not by 100 μM gliotoxin, 10 μM NEM or 40 μM 4,4′ DDP. Lane A: untreated; Lane B: 1 μM dexamethasone; Lane C: 3 μM gliotoxin; Lane D: 100 μM gliotoxin; Lane E: 10 μM NEM; Lane F: 40 μM 4,4′ DDP. All measurements at 6 h

DDP. The bottom traces indicate that, although the response of FIP-18 to 4,4′ DDP is a little slower than INDO-1, essentially the same calcium rise occurs over the first 10 min. In contrast, the calcium rise reported by FIP-18 in response to thapsigargin is significantly different to that reported by INDO-1 over the first 10 min. This difference is maintained over 40 min (data not shown).

Discussion

The exposure of PS during apoptosis is a well recognised event and is widely used as a diagnostic method for assessing apoptosis in cells. The mechanism of PS exposure has been shown to involve changes in those enzymes responsible for maintaining the asymmetry of the plasma membrane. These include an aminophospholipid transferase and a scramblase.[16] Evidence has been presented that it is modulation of both of these enzymes that is responsible for PS exposure during apoptosis.[12] Calcium can result in activation of the scramblase in eyrthrocytes and this has led to the suggestion that PS exposure in apoptosis may be the result of increased calcium rises preceding DNA fragmentation.[31]

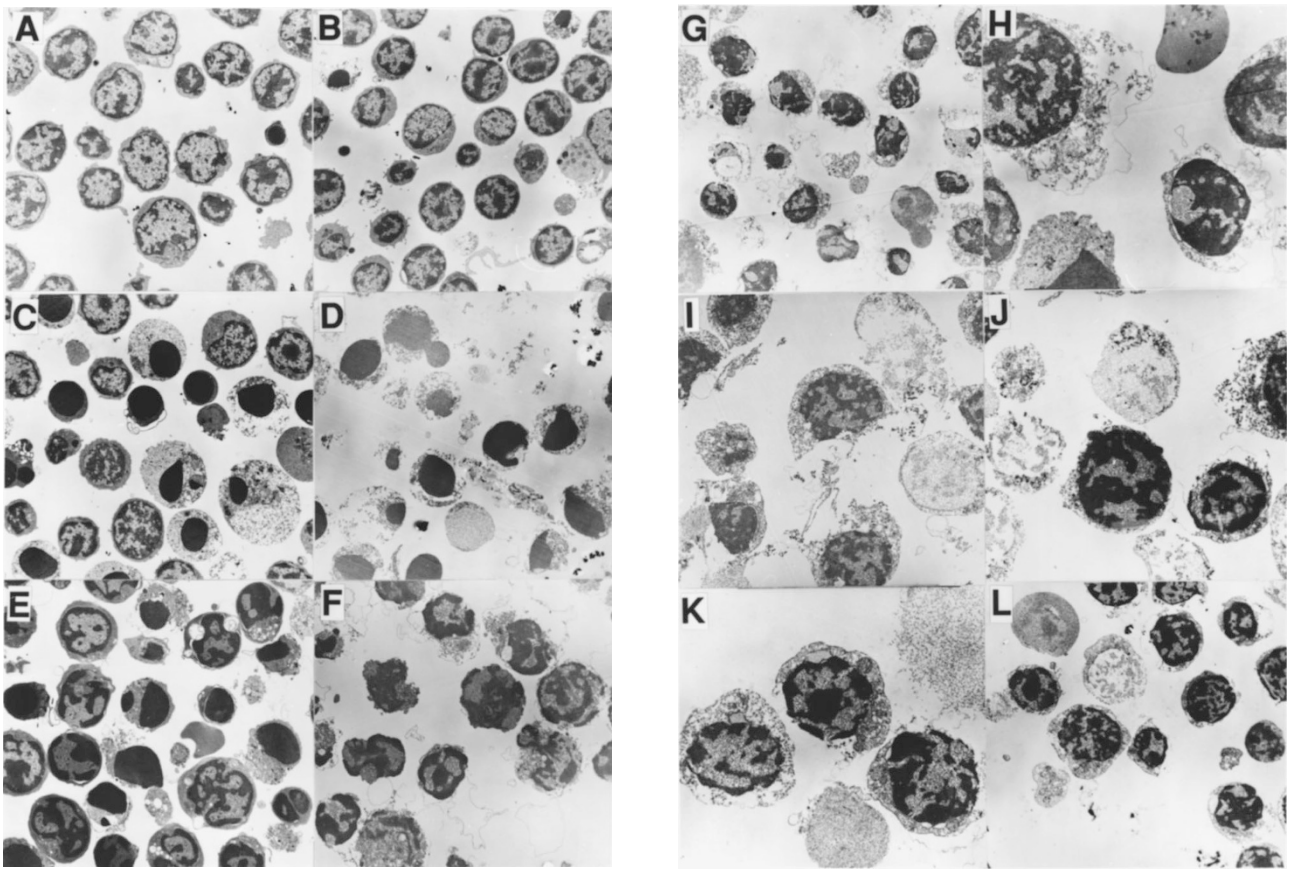

Figure 11 Morphology of thymocytes treated with gliotoxin, dexamethasone, NEM, 2,2′ and 4,4′ DDP. (**A**) Untreated 6 h. (**B**) Untreated 24 h. (**C**) 1 μM dexamethasone 6 h. (**D**) 1 μM dexamethasone 24 h. (**E**) 3 μM gliotoxin 6 h. (**F**) 100 μM gliotoxin 6 h. All $\times$2500. (**G**) 10 μM NEM 6 h. $\times$2500. (**H**) 10 μM NEM 6 h. (**I**) 3 μM 2,2′ DDP 6 h. (**J**) 3 μM 2,2′ DDP 24 h. (**K**) 40 μM 4,4′ DDP 6 h. (**L**) 40 μM 4,4′ DDP 24 h. All other panels $\times$6000

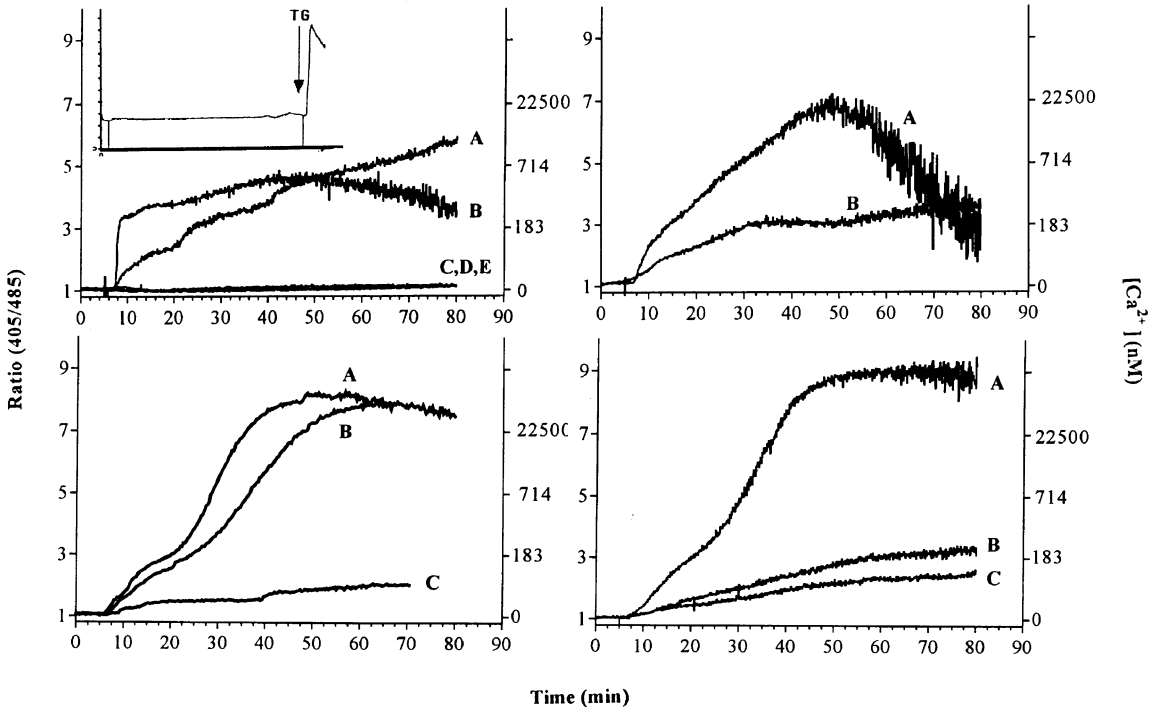

Figure 12 Calcium rises induced in thymocytes by gliotoxin, dexamethasone, NEM, 4,4′ DDP, 2,2′ DDP and DTNB. Left hand Y axis is Indo-1 405/485 ratio change; right hand Y axis is calculated calcium change; X axis is time. Top left panel (A) 100 μM gliotoxin; (B) 10 nM thapsigargin; (C) 1 μM dexamethasone; (D) 3 μM gliotoxin; (E) 100 μM DTNB. Inset: top left panel: No change in intracellular calcium in thymocytes treated with 1 μM dexamethasone for 2.5 h. Large calcium rise indicated at arrow due to addition of 10 nM thapsigargin at 2.5 h. Top right panel, (A) 50 μM NEM; (B) 10 μM NEM. Bottom left panel, (A) 5 μM 4,4′ DDP; (B) 1 μM 4,4′ DDP; (C) 0.5 μM DDP. Bottom right panel, (A) 5 μM 2,2′ DDP; (B) 1 μM 2,2′ DDP; (C) 0.5 μM 2,2′ DDP

We began this study in order to examine the relationship between any observed calcium rises in cells undergoing apoptosis and annexin V positive cells as a measure of PS exposure. We have demonstrated in the past that neither dexamethasone nor low concentrations of gliotoxin results in early (within 80 min) increases in calcium levels in thymocytes despite inducing extensive apoptosis in these cells within 4 h. We have now also shown that there is no detectable increases in intracellular calcium in thymocytes treated with dexamethasone up to 2.25 h. This is the time at which the $PS^{+}PI^{-}$ population is at a maximum in dexamethasone treated thymocytes. Moreover the kinetics of induction of apoptosis by thapsigargin, which causes rapid and sustained rises in calcium within minutes, shows that both induction of fragmentation of DNA and PS exposure are delayed compared to dexamethasone treated cells. Finally thapsigargin which rapidly raises intracellular calcium and inhibits DNA fragmentation in thymocytes treated with dexamethasone, was effective in inhibiting PS exposure induced by dexamethasone. These data support the idea that PS exposure is not due to any calcium rises *per se* but correlates with and precedes DNA fragmentation independent of the inducer of apoptosis.

We also observed that a class of compounds that do raise intracellular calcium and result exclusively in necrosis in thymocytes also cause an early (propidium iodide uptake) exposure of PS on the cell surface. It is significant that these agents, NEM, 2,2′ DDP, 4,4′ DDP and gliotoxin at 100 μM, in addition to inducing calcium rises, are all capable of interacting with reactive thiols on proteins. In fact we have shown that the calcium rise induced in thymocytes by gliotoxin and its simple analogs at high concentrations may be due to interaction of the toxin with plasma membrane protein thiols (Hurne, Waring *et al*, in preparation) although the nature of this thiol(s) is currently unknown. Previously 2,2′ DDP has also been shown to mobilise calcium from intracellular stores.[32] We cannot exclude the possibility that these agents interact with a variety of other accessible thiol groups on proteins.

It is significant that both scramblase activity, which results from increased calcium levels, and inhibition of the aminophospholipid transferase are required for maximal exposure of PS.[12] The aminophospholipid transferase has been demonstrated to be sensitive to thiol specific compounds and we would suggest that the increased exposure of PS due to NEM, 2,2′ DDP, 4,4′ DDP and gliotoxin at 100 μM is due to the simultaneous inhibition of the aminophospholipid transferase by thiol modification and activation of the scramblase due to calcium rises. These changes in enzyme activity may occur before membrane damage and subsequently lead to exclusive necrotic cell death. Unlike PS exposure in apoptosis, PS exposure in

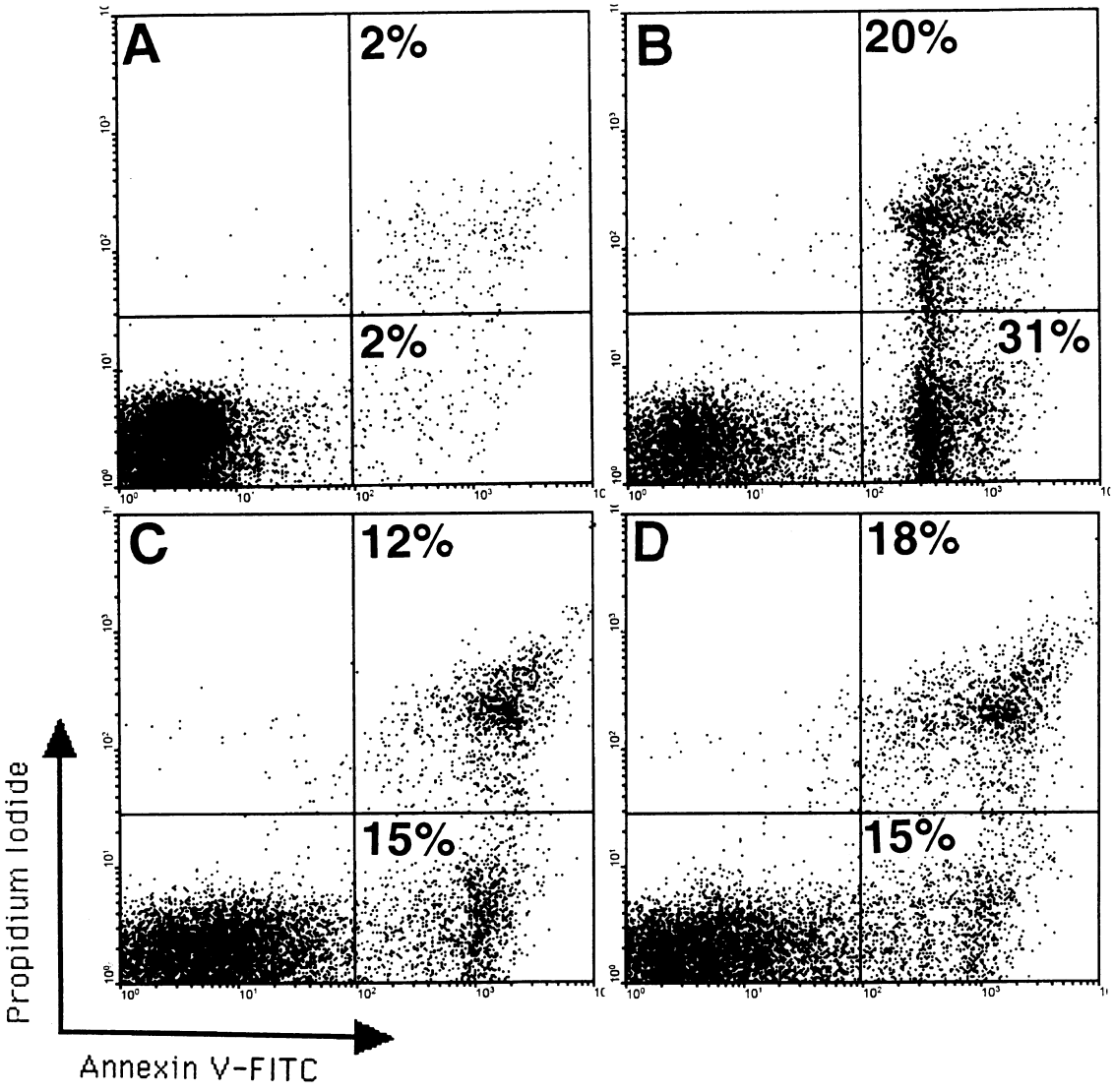

Figure 13 Co-incubation with thapsigargin abrogates PS exposure induced by dexamethasone at 6 h in thymocytes. (**A**) Untreated. (**B**) 1 μM dexamethasone. (**C**) 25 nM thapsigargin. (**D**) 1 μM dexamethasone+25 nM thapsigargin. Similar results were seen with 10 nM thapsigargin

this instance is not abrogated by removal of extracellular calcium.

PS^+PS^- exposure by these agents may not strictly correlate with calcium rises. Thus at 10 μM 4,4′ DDP, PS exposure is just evident although 3 μM 4,4′ DDP produced a maximal calcium rise although 2,2′ DDP at 3 μM did produce a substantial population of PS^+PI^- cells. This could be explained by the requirement for concomitant inhibition of aminophospholipid transferase and a calcium ion increase. NEM is known to inhibit the transferase[33,34] but we have as yet no information of the relative efficacy of 2,2′ or 4,4′ DDP in this respect. The fact that thapsigargin does not result in rapid induction of the same level of PS^+PI^- cells as does gliotoxin at 100 μM despite both producing significant calcium rises, could be due to the fact that thapsigargin is not obviously a thiol specific agent. Similarly the thiol specific agent DTNB, which has no effect on PS exposure, does not cause any calcium increase although its lack of effect may be due to increased polarity and lack of membrane penetration.

More significantly, the data in Figure 14 is consistent with the thiol specific agent 4,4′ DDP causing early, near membrane calcium rises. The calcium sensitive dye FIP-18 is essentially INDO-1 modified with a $C_{12}H_{26}$ tail which anchors the dye in the plasma membrane similar to the Fura-2 analog FFP-18.[35] In Figure 14, INDO-1, which would be expected to be distributed in all cellular compartments (although not necessarily evenly) including near the membrane, reports the same initial calcium rise as FIP-18 anchored in the membrane. The different calcium rises induced by thapsigargin on the other hand are consistent with no or little near membrane calcium rise

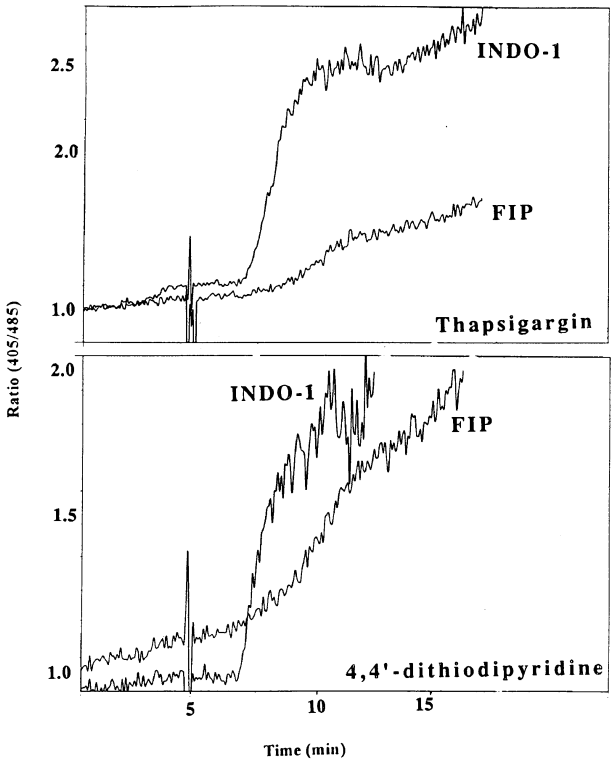

Figure 14 Initial calcium rises induced in thymocytes by thapsigargin (top panel) or 4,4′ DDP (bottom panel) monitored by either INDO-1 or FIP-18

initially. This is consistent with the mode of action of thapsigargin which causes a calcium rise by blocking the action of the endoplasmic reticulum calcium uptake pump. This then stimulates capacitative filling of the intracellular pools. Although it is not clear how this occurs, calcium entry induced by thapsigargin is closely coupled to the filling of the intracellular stores and might not be expected to give rise to large near membrane concentrations of free calcium. This could account for rapid activation of the scramblase in the case of 4,4′ DDP and other thiol specific agents but not by thapsigargin. We have presented evidence that thapsigargin induces apoptosis by damaging mitochondria.[24] Cell killing by the thiol specific agents may involve overt damage due to calcium rises including near membrane rises e.g. by activation of phospholipases or proteases and be independent of the PS exposure. Importantly overt membrane damage by these agents as assessed by propidium iodide uptake, precedes the calcium rises and PS exposure as assessed by annexin V-FITC.

In conclusion our data suggests that detectable calcium rises do not play an important part in dexamethasone or gliotoxin induced PS exposure. Apoptosis has been shown to be generally associated with loss of glutathione[30,36] which would presumably place the cell under oxidative stress resulting in modification of protein thiols including those on the aminophosphotransferase. Whether this is sufficient to cause PS exposure in the absence of significant intracellular calcium rises awaits further investigation.

Importantly the generality of PS exposure prior to PI uptake as a diagnostic sign of apoptosis rather than necrosis breaks down in this study and the use of PS exposure alone as a measure of apoptosis, even after gating out PI positive cells, should be avoided without independent corroborative evidence.

Materials and Methods

Chemicals

All chemicals were obtained from sigma and were the purest available. INDO-1 was purchased from Molecular Probes Inc, Eugene, Oregon, USA and FIP-18 AM from TefLabs, Austin, Texas, USA.

Cells

Thymocytes were harvested from 10 day-old Balb/c mice in F15 media with 5% FCS, passed through a stainless steel mesh, filtered through Nybolt nylon monofilament gauze (60 micron) and centrifuged at $300 \times g$ for 5 min at 0°C. Cells were always >95% viable after harvest otherwise they were discarded. Cells were suspended in fresh F15 media with 5% FCS at 1×10^6/ml unless otherwise stated. During treatments cells were incubated in 24 well plates or in culture flasks at 37°C and 5% CO_2.

DNA fragmentation

DNA fragmentation was estimated by propidium iodide staining of ethanol fixed cells as previously described.[24] Cells were pelleted and fixed in 70% ethanol overnight. Cells were then washed three times in PBS and treated with propidium iodide (final 40 μg/ml) and RNAase (final 10 μg/ml) for at least 30 min before FACS analysis. The subdiploid population of cells was taken as the fraction of apoptotic cells. Analysis was performed using WinMDI kindly supplied by Joseph Trotter, Scripps Institute, La Jolla, CA, USA. All graphed data is the mean of at least two separate incubations. Histograms or dot plots are typical of at least two separate experiments.

Annexin V assay

Phosphatidylserine exposure was assessed with FITC conjugated Annexin V using an Oncogene Research Products Apoptosis Detection Kit according to the manufacturer's instructions except that all recommended volumes were reduced by 25%. This did not affect the results but resulted in significant saving of reagent. Briefly, treated cells were adjusted to 1×10^6/ml and 0.5 ml transferred to a FACS tube. Cells were washed twice in cold PBS and gently resuspended in 125 μl of cold 1× Binding Buffer containing 0.3 μl Annexin V-FITC solution. Cells were incubated for 15 min in the dark followed by the addition of 125 μl of cold Binding Buffer containing 2.5 μl of propidium iodide (stock 30 μg/ml). Samples were then placed on ice in the dark and analyzed immediately.

Calcium

Intracellular calcium levels were measured using the INDO-1 method as previously described.[24] Thymocytes were treated with INDO-1 AM at 0.3 μg/ml at 37°C at 2×10^7/ml in 400 μl for 15–20 min in F15 with 1% FCS. After loading with dye, 100 μl of the

cells were diluted into 1.9 ml of F15 followed by immediate analysis using a Becton Dickinson FACSSTAR instrument. Extracellular calcium was normally present at 1.3 mM. Data was collected at the rate of 100–200 cells/s. Typically 400 000–600 000 events were collected. Data was collected for 30–180 min depending on the experiment. Fluorescence at 405 and 485 nM and the 405/485 ratio were monitored. The 405/485 ratio (R) was set to a value of 1 at channel 100 before each run. The ratio was monitored for exactly 5 min before rapid (<10 s) addition of the agent under study. The protocol for using FIP-18 AM was identical to INDO-1 except that the cells were incubated for 2 h with FIP-18 acetoxymethyl ester (AM) instead of 15–20 min. Values of Rmax and Rmin were calculated as described[24] for each experiment. Intracellular calcium changes were calculated using $[Ca^{2+}]i=Kd \times \{[R-Rmin]/[Rmax-R]\} \times Sf2/Sb2$ and the calcium scale labelled accordingly. Values of Rmax were typically 7–8.5 and Rmin 0.12–0.17.

Agarose gel electrophoresis

Cells were pelleted after treatment and suspended in 1 ml PBS and 1 ml 2$\times$ neutral lysing buffer (0.2 M NaCl, 10 mM EGTA, 20 mM Tris-HCL, 1% SDS, pH 8) and 50 μl pronase solution (10 mg/ml). After overnight incubation at 37°C cells were extracted using phenol/chloroform and the DNA precipitated from the aqueous solution using 70% ethanol at -78°C. DNA was pelleted and washed three times in ice cold ethanol, dried and rehydrated in 10 mM EDTA. Electrophoresis was carried out using 2% agarose gels and bands viewed on a UV transilluminator.

Electron microscopy

Apoptosis or necrosis was confirmed using morphological examination of cells using EM as previously described.[24] Cells were pelleted after treatment and washed once in PBS pelleted again and fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4), stained with lead citrate and uranyl acetate. Sections were cut and viewed on a Phillips 301 electron microscope.

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