

Research article

## Deciphering the plasma membrane hallmarks of apoptotic cells: Phosphatidylserine transverse redistribution and calcium entry

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### Abstract

**Background:** During apoptosis, Ca<sup>2+</sup>-dependent events participate in the regulation of intracellular and morphological changes including phosphatidylserine exposure in the exoplasmic leaflet of the cell plasma membrane. The occurrence of phosphatidylserine at the surface of specialized cells, such as platelets, is also essential for the assembly of the enzyme complexes of the blood coagulation cascade, as demonstrated by hemorrhages in Scott syndrome, an extremely rare genetic deficiency of phosphatidylserine externalization, without other apparent pathophysiologic consequences. We have recently reported a reduced capacitative Ca<sup>2+</sup> entry in Scott cells which may be part of the Scott phenotype.

**Results:** Taking advantage of these mutant lymphoblastoid B cells, we have studied the relationship between this mode of Ca<sup>2+</sup> entry and phosphatidylserine redistribution during apoptosis. Ca<sup>2+</sup> ionophore induced apoptosis in Scott but not in control cells. However, inhibition of store-operated Ca<sup>2+</sup> channels led to caspase-independent DNA fragmentation and decrease of mitochondrial membrane potential in both control and Scott cells. Inhibition of cytochrome P450 also reduced capacitative Ca<sup>2+</sup> entry and induced apoptosis at comparable extents in control and Scott cells. During the apoptotic process, both control and more markedly Scott cells externalized phosphatidylserine, but in the latter, this membrane feature was however dissociated from several other intracellular changes.

**Conclusions:** The present results suggest that different mechanisms account for phosphatidylserine transmembrane migration in cells undergoing stimulation and programmed death. These observations testify to the plasticity of the plasma membrane remodeling process, allowing normal apoptosis even when less fundamental functions are defective.

### Background

During apoptosis, changes in cytosolic Ca<sup>2+</sup> are likely to play a critical role by triggering the activation of Ca<sup>2+</sup>-dependent events inducing global intracellular and mor-

phological modifications [1,2]. Among these changes, early transverse redistribution of plasma membrane phosphatidylserine is one of the well-documented hallmarks of cells undergoing apoptosis [3,4], and has been

shown to depend on caspase-3 protease activity [5] and cytoplasmic  $\text{Ca}^{2+}$  concentration [6,7]. The resulting exposure of phosphatidylserine in the outer leaflet may serve as a recognition signal for phagocytosis of senescent cells to be rapidly cleared [3,8–10]. Phosphatidylserine is also essential for the assembly of the blood coagulation enzyme complexes at the surface of stimulated specialized cells such as platelets, as demonstrated by hemorrhages in Scott syndrome, an extremely rare inherited bleeding disorder transmitted as an autosomal recessive trait [11–13]. Blood cells from these patients show a defective membrane-associated procoagulant activity due to a decreased ability of phosphatidylserine externalization after stimulation [11,12], suggesting that Scott phenotype results from the deletion or mutation of a regulatory element involved in this lipid transverse transport [12,14]. Whether phosphatidylserine transmembrane migration is governed by common mechanism(s) in apoptotic cells or stimulated platelets remains uncertain. Last results suggest that apoptosis and procoagulant stimulation operate through different pathways to activate phosphatidylserine externalization mediated by a common molecular entity [15].

In non-excitabile cells, one of the pathways for the regulation of intracellular  $\text{Ca}^{2+}$  concentration involves store-operated  $\text{Ca}^{2+}$  influx (also referred to as capacitative  $\text{Ca}^{2+}$  entry). Activation of voltage-independent  $\text{Ca}^{2+}$  channels after sustained depletion of intracellular  $\text{Ca}^{2+}$  stores accounts for store-operated  $\text{Ca}^{2+}$  entry [16]. It was originally postulated that store-operated  $\text{Ca}^{2+}$  entry function is limited to replenishment of intracellular  $\text{Ca}^{2+}$  stores, several observations however provide evidence for an important role played by these channels in cell homeostasis or death [17–20]. The significance of capacitative  $\text{Ca}^{2+}$  entry in the development of apoptosis has been shown in hamster embryo cells. In this model, a reduced store-operated  $\text{Ca}^{2+}$  entry correlates with a dysregulation of intracellular vesicular traffic and results in cell death [19]. Capacitative  $\text{Ca}^{2+}$  entry is also defective in immunodeficiency characterized by a lack of lymphocytes proliferation [17,21]. In addition, SKF 96365, a commonly used store-operated  $\text{Ca}^{2+}$  channel inhibitor, is a potent inducer of apoptosis in HL60 cells [20] suggesting that store-operated  $\text{Ca}^{2+}$  entry is an important regulator of the induction and execution phases of apoptosis.

We have previously reported that EBV-transformed B cells from a patient with Scott syndrome show a defective store-operated  $\text{Ca}^{2+}$  entry after stimulation [22], which enabled further investigation of the link between this mode of  $\text{Ca}^{2+}$  entry and phosphatidylserine transmembrane redistribution [23]. Scott patients do not present other clinical symptoms than bleeding episodes, in par-

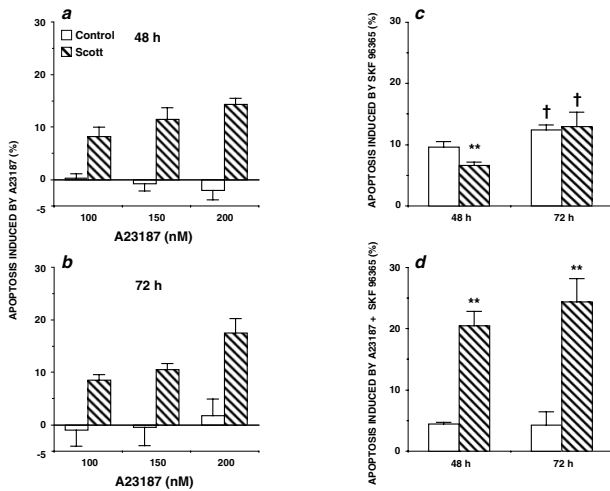
ticular (auto)immune disorders have not been observed [11,12], suggesting that apoptosis and clearance of cell fragments are normal although procoagulant phosphatidylserine exposure is impaired.

Taking advantage of these mutant lymphoblastoid B cells, in which rapid phosphatidylserine externalization is considerably reduced following drastic stimulation by  $\text{Ca}^{2+}$  ionophore, but nevertheless able to undergo apoptosis over a longer time period [12,14], the present study is aiming at establishing a relationship between store-operated  $\text{Ca}^{2+}$  entry and phosphatidylserine redistribution during apoptosis. For this, several agents were used,  $\text{Ca}^{2+}$  ionophore A23187 which induces  $\text{Ca}^{2+}$  release and entry [24], the  $\text{Ca}^{2+}$  channel blocker SKF 96365 [25] and the cytochrome P450 inhibitor SKF 525A which also inhibits store-operated  $\text{Ca}^{2+}$  entry in platelets [26]. Inhibition of store-operated  $\text{Ca}^{2+}$  channels by SKF 96365 induces caspase-independent DNA fragmentation and decrease of mitochondrial membrane potential in both control and Scott cells, while  $\text{Ca}^{2+}$  influx following treatment by  $\text{Ca}^{2+}$  ionophore was only protective in control cells. The dissociation of phosphatidylserine transverse migration from other intracellular changes indicates the existence of alternative pathways for the accomplishment of basic functions or responses involving the remodeling of the cell plasma membrane.

## Results

### **Apoptosis and store-operated $\text{Ca}^{2+}$ entry**

Lymphoblasts were treated for 48 or 72 h with the  $\text{Ca}^{2+}$  ionophore A23187 at concentrations of 100, 150 and 200 nM (Figs. 1a and 1b). Control cells showed normal proportions of DNA PI staining, corresponding to normal 2N or 4N DNA content; A23187 even exerted a somewhat protective effect against basal apoptosis. At the opposite, in Scott B cells cytometric analysis revealed nuclei with low DNA staining (sub-G1 peak) corresponding to apoptotic cells. The percentage of apoptotic nuclei was A23187 concentration-dependent and at 200 nM,  $17.6 \pm 2.7$  % of the cells have hypodiploid DNA after 72 h. The apoptotic character of A23187-treated lymphoblasts was confirmed by TUNEL assay (see below). A reduced capacitative  $\text{Ca}^{2+}$  entry has been shown to correlate with apoptosis [19]. Recently, we have reported that Scott lymphoblasts show a defective store-operated  $\text{Ca}^{2+}$  entry [22]. Therefore, it was interesting to assess whether inhibition of capacitative  $\text{Ca}^{2+}$  channels could induce apoptosis in control B lymphoblasts. The experiments were performed in the presence of 10  $\mu\text{M}$  SKF 96365, a blocker of store-operated  $\text{Ca}^{2+}$  channels [25]. At 10  $\mu\text{M}$ , SKF 96365 reduced by approximately 30 % the level of capacitative  $\text{Ca}^{2+}$  entry induced by thapsigargin (1  $\mu\text{M}$ ) in either control or Scott cells (not shown). As shown in Fig.

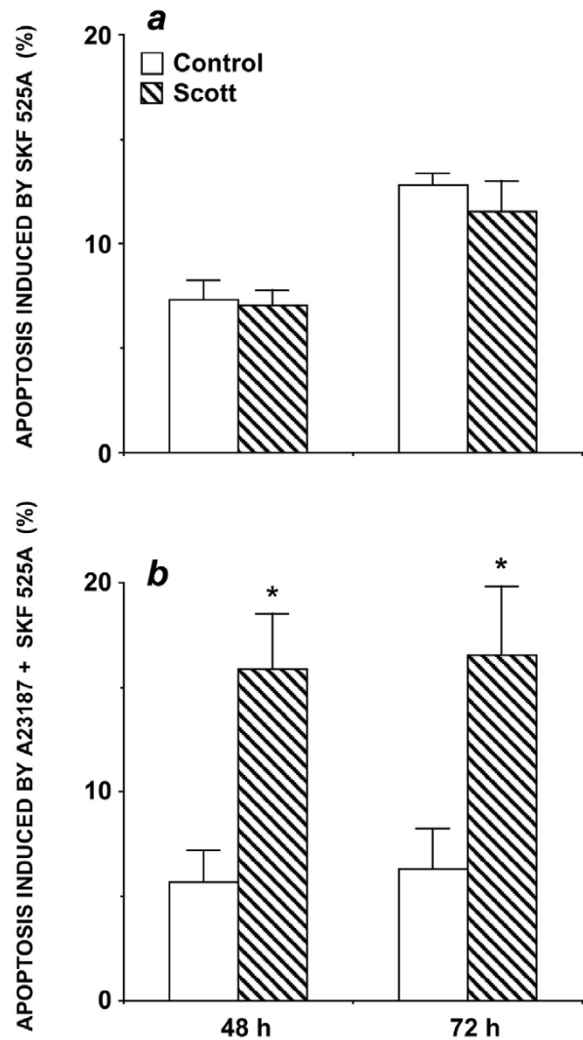


**Figure 1**  
Effect of calcium ionophore A23187 and/or calcium channel inhibitor SKF 96365 on cell death. Control and Scott B lymphoblasts were treated with A23187 (100–200 nM) for 48 (a) or 72 (b) h, or with SKF 96365 (10 μM) for 48 or 72 h in the absence (c) or in the presence of calcium ionophore A23187 (200 nM) (d). The degree of apoptosis was determined by measurement of hypodiploid DNA using propidium iodide (0.1 mg/ml). Values are mean ± SEM of at least 8 independent determinations. \*\**P* < 0.01 versus control cells. †*P* < 0.05 versus the corresponding values at 48 h of treatment.

1c, the same concentration of SKF 96365, was able to induce apoptosis in both control and Scott cells.

After 48 h, SKF 96365-induced apoptosis in control cells was significantly higher than in Scott cells (9.6 ± 0.9 % and 6.6 ± 0.6 % in control and Scott cells, respectively; *P* < 0.01), however, after 72 h of treatment, SKF 96365 induced apoptosis at comparable degrees in both types of cells (12.4 ± 0.8 % and 12.9 ± 2.4 % in control and Scott cells, respectively). Higher concentrations of SKF 96365 were responsible for necrosis in both types of cells. It should be noted that when cells were treated with the combination of Ca<sup>2+</sup> ionophore A23187 + SKF 96365, the degrees of apoptosis were almost additive at 48 h, and ~40 % higher at 72 h in Scott cells with respect to values obtained in the presence of A23187 alone, whereas A23187 remained protective in control lymphoblasts (Figure 1d).

Some studies have shown that cytochrome P450 activity is critical with respect to the regulation of store-operated Ca<sup>2+</sup> influx [27,28]. To verify whether cytochrome P450 pathway is implicated in Ca<sup>2+</sup> entry induced by depletion of Ca<sup>2+</sup> stores in EBV lymphoblasts, experiments were also performed in the presence of 10 μM SKF 525A, a cytochrome P450 inhibitor [28]. As observed with SKF



**Figure 2**  
Effect of the cytochrome P450 inhibitor SKF 525A on cell death. Control and Scott B lymphoblasts were treated with SKF 525A (10 μM) for 48 or 72 h in the absence (a) or in the presence of calcium ionophore A23187 (200 nM) (b). The degree of apoptosis was determined by measurement of hypodiploid DNA using propidium iodide (0.1 mg/ml). Values are mean ± SEM of at least 6 independent determinations. \**P* < 0.05 versus control cells.

96365, SKF 525A reduced capacitative Ca<sup>2+</sup> entry induced by thapsigargin (1 μM) by approximately 30 % in either control or Scott cells (not shown). In addition, after 48 or 72 h of treatment, 10 μM SKF 525A induced apoptosis at comparable extents in control and Scott cells (Fig. 2a). When cells were treated with the combination of Ca<sup>2+</sup> ionophore A23187 + SKF 525A, the degrees of apoptosis in Scott cells were not significantly different from those observed in the presence of A23187 alone (Fig. 2b). Again, A23187 exerted a protective effect

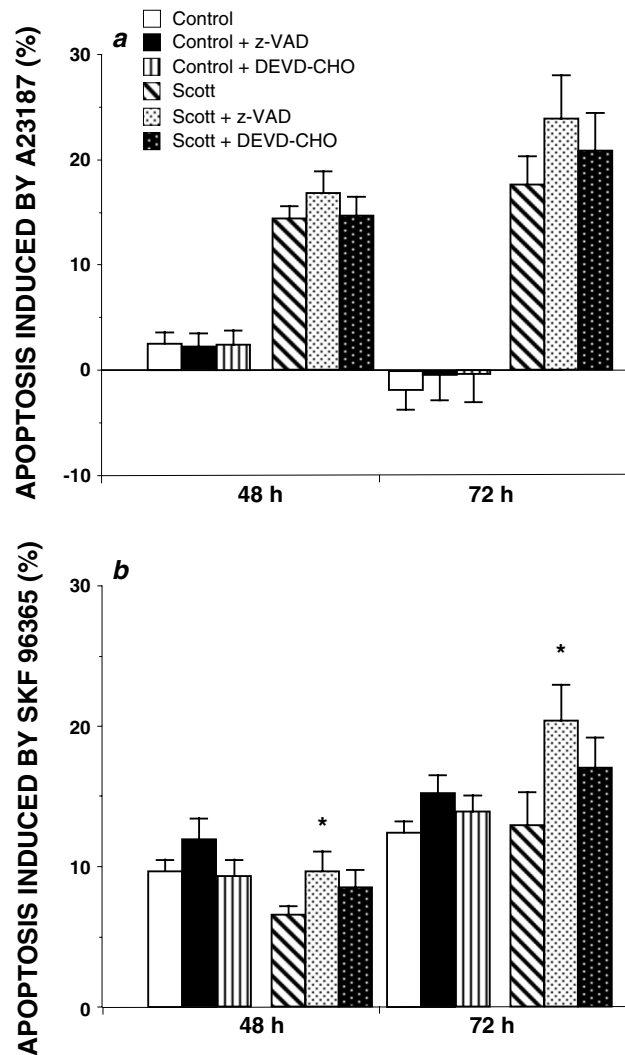
against SKF 525A-induced apoptosis in control lymphoblasts, more pronounced at 72 h (Fig. 2b).

Experiments using the extracellular  $\text{Ca}^{2+}$  chelator EGTA (1–2 mM) were designed in order to evaluate the relationship between store-operated  $\text{Ca}^{2+}$  entry and apoptosis. At 1 mM, EGTA did not induce apoptosis nor affected  $\text{Ca}^{2+}$  ionophore-evoked apoptosis. However, higher concentrations of EGTA (1.5 and 2 mM) enhanced cell mortality by necrosis, making measurements of the degree of apoptosis almost impossible (not shown).

To determine whether caspases are involved in this apoptotic process,  $\text{Ca}^{2+}$  ionophore- or SKF 96365-treated cells were incubated in the absence or presence of the broad-spectrum caspase inhibitor z-VAD.fmk (50  $\mu\text{M}$ ) or the more specific caspase-3 inhibitor, Ac-DEVD-CHO (50  $\mu\text{M}$ ) [29]. Apoptosis analysis, assessed by PI staining, showed that none of these inhibitors had significant effect on DNA fragmentation after 48 or 72 h of A23187 treatment of either control or Scott cells (Fig. 3a). However, z-VAD.fmk interfered in the effect of SKF 96365 on Scott cells, even exerting an apparent paradoxical potentiation of apoptosis, whereas it did not affect the fate of control cells to a significant extent (Fig. 3b).

In order to assess the effects of  $\text{Ca}^{2+}$  ionophore and channel blockers on apoptosis,  $\text{Ca}^{2+}$  signaling was monitored in viable cells using Fluo-3 fluorochrome and PI to define a gate excluding necrotic cells, i.e. PI positive cells representing less than 10 % of the whole population.

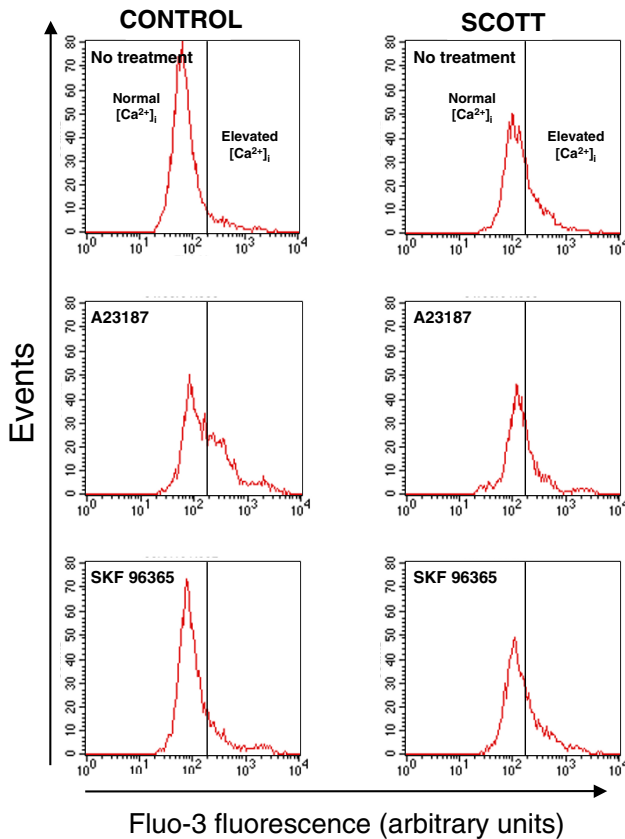
Under control condition (Fig. 4), approximately 95 % of viable cells presented "normal"  $[\text{Ca}^{2+}]_i$  ( $68 \pm 6$  and  $78 \pm 14$  fluorescence arbitrary units in control and Scott cells respectively) and only  $3 \pm 1$  % of cells showed elevated  $[\text{Ca}^{2+}]_i$  (Table 1). After A23187 (200 nM) treatment, the latter population with elevated  $[\text{Ca}^{2+}]_i$  was significantly increased as well as  $[\text{Ca}^{2+}]_i$  itself, but this occurred in control cells only, and the effect was not inhibited by the caspase-3 inhibitor Ac-DEVD-CHO (Table 1). In addition, SKF 96365 did not modify either  $[\text{Ca}^{2+}]_i$  or number of events in both types of cells. Similar results were obtained with SKF 525A (not shown). However, it should be noted that when cells were treated with the combination of  $\text{Ca}^{2+}$  ionophore A23187 + SKF 96365,  $[\text{Ca}^{2+}]_i$  was enhanced in control but not in Scott cells. Furthermore, in response to UV irradiation, both types of cells underwent apoptosis as determined by DNA fragmentation but  $[\text{Ca}^{2+}]_i$  was not modified. For example, at 5 J/cm<sup>2</sup>, 23  $\pm$  3 % and 28  $\pm$  5 % of the normal and Scott cells had hypodiploid DNA, respectively.



**Figure 3**  
Effect of the caspase inhibitors z-VAD.fmk or Ac-DEVD-CHO on cell death induced by calcium ionophore A23187 or calcium channel inhibitor SKF 96365. Control and Scott B lymphoblasts were treated with z-VAD.fmk (50  $\mu\text{M}$ ) or Ac-DEVD-CHO (50  $\mu\text{M}$ ) for 48 or 72 h in the presence of calcium ionophore A23187 (200 nM) (a), or in the presence of the calcium channel inhibitor SKF 96365 (10  $\mu\text{M}$ ) (b). The degree of apoptosis was determined by measurement of hypodiploid DNA using propidium iodide (0.1 mg/ml). Values are mean  $\pm$  SEM of at least 8 independent determinations. \* $P < 0.05$  versus the corresponding value in the absence of caspase inhibitor.

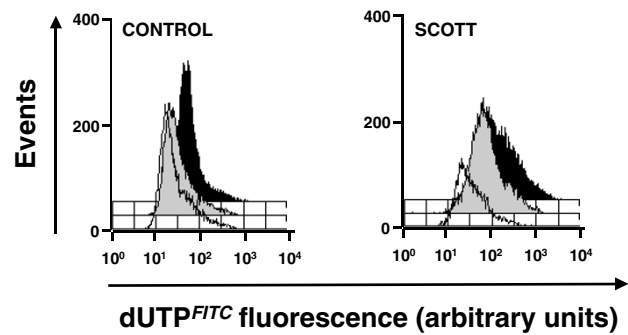
#### DNA fragmentation and mitochondrial membrane potential

A switch from apoptosis to necrosis has been described in B lymphocytes after incubation in the presence of caspase inhibitor [30]. In order to assess that each of A23187 or SKF 96365 induces apoptosis and not necrosis, TUNEL assay was performed and changes of mito-



**Figure 4**  
 $[Ca^{2+}]_i$  determination in control and Scott cells by flow cytometry. Cells treated for 72 h by 200 nM A23187 or 10  $\mu$ M SKF 96365 were loaded with the fluorochrome Fluo-3 (3  $\mu$ M) and propidium iodide (PI, 10  $\mu$ g/ml). Histograms show the number of cells as a function of Fluo-3 fluorescence for the PI negative cell population only. One representative experiment out of four performed likewise is shown.

chondrial membrane potential were measured by flow cytometry. Early in apoptosis, TUNEL labeling and mitochondrial membrane permeability increase and the mitochondrial inner transmembrane potential decreases [31]. Fig. 5 shows TUNEL positive staining in SKF 96365-treated cells but not in A23187-treated control cells, and in both  $Ca^{2+}$  ionophore- and SKF 96365-treated Scott cells after 48 h, confirming PI results. Similar observations were made after 72 h treatment (not shown). While in control cells, A23187 induced a non-significant decrease of the JC-1 red/green fluorescence ratio (reflecting  $\Delta\psi$ ), in Scott cells the effect was more pronounced, corresponding to higher apoptosis levels (Table 2). As observed by PI analysis, SKF 96365 treatment induced comparable decreases of the JC-1 red/green fluorescence ratio in both types of cells after 48 or 72 h treatment (Table 2). z-VAD.fmk treatment limited the SKF 96365-induced decrease in JC-1 fluorescence af-



**Figure 5**  
 Calcium ionophore and SKF 96365-induced apoptosis of control and Scott B lymphoblasts, assessed by TUNEL and flow cytometry. Control and Scott lymphoblasts were treated with A23187 (200 nM, gray) or SKF 96365 (10  $\mu$ M, black) for 48 h, and then stained for DNA fragmentation by TUNEL as described in the "Experimental Procedures". Fluorescence shifts observed with A23187 or SKF 96365 treatment were compared to those obtained with non-treated cells (empty). Results were reproduced in at least 3 different experiments.

ter 48 h treatment, but had no effect on the reduction of JC-1 fluorescence ratio corresponding to A23187 or SKF 96365 after 72 h treatment.

#### Phosphatidylserine externalization

Apoptotic cells were assessed for phosphatidylserine exposure by functional procoagulant prothrombinase assay, and by annexin V labeling. After  $Ca^{2+}$  ionophore or SKF 96365 treatment, the degree of phosphatidylserine externalization remained weak at 48 h and was not affected by the caspase inhibitor z-VAD.fmk (Fig. 6a). At 72 h of A23187 or SKF 96365 treatment, the degree of phosphatidylserine exposure was enhanced in both types of cells, and more significantly in SKF 96365-treated cells ( $P < 0.05$ ). Phosphatidylserine externalization was not affected by the caspase inhibitor z-VAD.fmk in control cells. In Scott cells, z-VAD.fmk however prevented the phosphatidylserine redistribution induced by  $Ca^{2+}$  ionophore ( $P < 0.05$ ), but did not reduce the effect of SKF 96365, even exerting an apparent paradoxical potentiation of phosphatidylserine externalization probably due to a membrane destabilizing effect when used at 50  $\mu$ M [32] (Fig. 6b).

In order to compare with more drastic and rapid stimulation, it should be noted that after 10 min of A23187 treatment at 1  $\mu$ M, prothrombinase activity was enhanced in both type of cells, but as previously reported [12,22] B lymphoblasts from the Scott patient displayed a ~60% reduction of prothrombinase activity when compared with control cells (Fig. 6c). Consistent with the

**Table 1: Changes of intracellular  $Ca^{2+}$  concentration in the cell population with elevated  $[Ca^{2+}]_i$ , assessed by flow cytometry using Fluo-3 fluorescence (arbitrary units) in control and Scott cells after 48 or 72 h of apoptosis induction by calcium ionophore A23187 (200 nM) or/and SKF 96365 (10  $\mu$ M), in the absence and presence of the caspase inhibitor Ac-DEVD-CHO (50  $\mu$ M). Values are the mean  $\pm$  SEM of 4 independent experiments.**

CELLS	No treatment	A23187 (200 nM)	SKF 96365 (10 $\mu$ M)	A23187 + SKF 96365	Ac-DEVD-CHO + A23187
Control (48 h)	398 $\pm$ 25	545 $\pm$ 51**	409 $\pm$ 17	524 $\pm$ 25*	522 $\pm$ 23*
Scott (48 h)	371 $\pm$ 5	382 $\pm$ 1	390 $\pm$ 7	371 $\pm$ 14	380 $\pm$ 10
Control (72 h)	361 $\pm$ 22	470 $\pm$ 25*	417 $\pm$ 31	490 $\pm$ 44*	520 $\pm$ 90*
Scott (72 h)	403 $\pm$ 62	408 $\pm$ 70	376 $\pm$ 46	354 $\pm$ 54	356 $\pm$ 43

\* $P < 0.05$ , \*\* $P < 0.01$  versus the corresponding values obtained with non-treated cells.

**Table 2: Changes of mitochondrial membrane potential, assessed by flow cytometry using JC-1 red/green fluorescence ratio in control and Scott cells after 48 or 72 h of apoptosis induction by calcium ionophore A23187 (200 nM) or SKF 96365 (10  $\mu$ M) in the absence and presence of the caspase inhibitor z-VAD.fmk (50  $\mu$ M). Values are the mean  $\pm$  SEM of 4 independent experiments.**

CELLS	No treatment	A23187 (200 nM)	SKF 96365 (10 $\mu$ M)	zVAD + A23187	zVAD + SKF 96365
Control (48 h)	1.37 $\pm$ 0.24	1.05 $\pm$ 0.16	0.85 $\pm$ 0.11*	1.16 $\pm$ 0.03	1.01 $\pm$ 0.09
Scott (48 h)	1.25 $\pm$ 0.20	1.01 $\pm$ 0.17	0.96 $\pm$ 0.20*	1.11 $\pm$ 0.08	1.20 $\pm$ 0.12
Control (72 h)	1.26 $\pm$ 0.09	1.1 $\pm$ 0.16	0.89 $\pm$ 0.06*	0.97 $\pm$ 0.03	0.88 $\pm$ 0.04*
Scott (72 h)	1.55 $\pm$ 0.12	1.02 $\pm$ 0.22*	1.04 $\pm$ 0.26*	0.97 $\pm$ 0.16*	0.89 $\pm$ 0.21*

\* $P < 0.05$  versus the corresponding values obtained with non-treated cells.

data obtained by the prothrombinase assay at 72 h of A23187 or SKF 96365 treatment (Fig. 6b) and  $[Ca^{2+}]_i$  determinations (Fig. 4), the number of annexin V-positive cells increased in both types of cells (Fig. 7). Moreover, upon induction of apoptosis by UV irradiation, control and Scott cells showed approximately the same proportion of annexin V-positive cells ( $\sim 31 \pm 5\%$  and  $34 \pm 6\%$  of control and Scott cells, respectively). Ac-DEVD-CHO did not prevent annexin V labeling (not shown), suggesting that phosphatidylserine externalization is independent of caspase-3 activity.

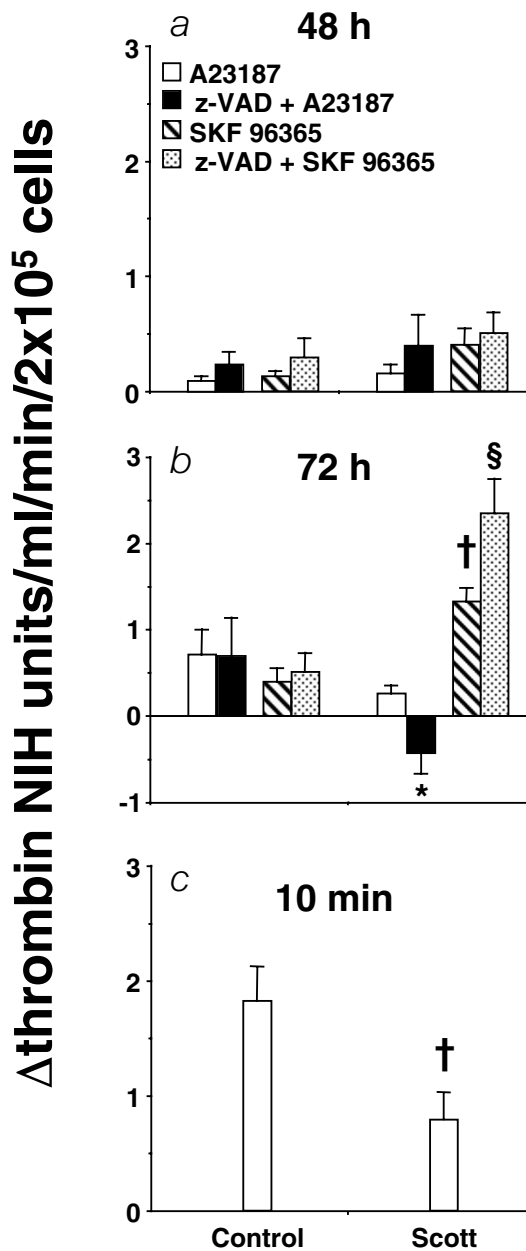
## Discussion

We have previously reported that Scott cells present a defect of phosphatidylserine externalization in association with a reduced store-operated  $Ca^{2+}$  entry after stimulation [22]. In hamster embryo cells, a reduced store-operated  $Ca^{2+}$  entry correlates with a dysregulation of intracellular vesicular traffic and apoptosis [19]. Here, we provide direct evidence that impairment of store-operated  $Ca^{2+}$  entry can causally be implicated in the development of apoptosis since  $Ca^{2+}$  influx protects control cells against apoptosis. In addition, Scott cells are able to ex-

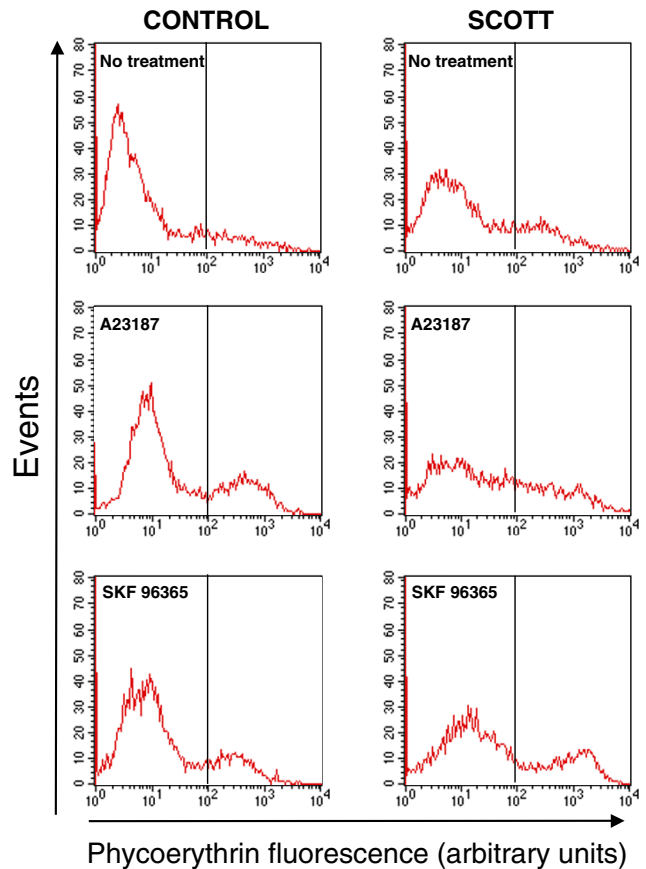
pose phosphatidylserine during apoptosis indicating that the mechanisms controlling membrane phosphatidylserine redistribution during apoptosis and procoagulant response are probably different.

$Ca^{2+}$  ionophore has been reported to induce  $Ca^{2+}$  release from intracellular stores, and after sustained depletion, voltage-independent  $Ca^{2+}$  entry across the plasma membrane [24]. In this study, A23187 treatment induced apoptosis only in Scott, but not in control cells, and an increase in  $[Ca^{2+}]_i$  only in the latter, indicating that  $Ca^{2+}$  exerts a protective effect against apoptosis. Because  $Ca^{2+}$  release from intracellular stores was similar in Scott and control cells [22], the present results suggest that reduced  $Ca^{2+}$  entry indeed correlates with apoptosis induction.

In T and B lymphocytes, it has been shown that stimulation of CD95, which leads to apoptosis, blocks store-operated  $Ca^{2+}$  channels and influx through the activation of acidic sphingomyelinase and ceramide release [33]. In our study, if capacitative  $Ca^{2+}$  entry is involved in the induction of apoptosis, direct inhibition of this particular



**Figure 6**  
 Procoagulant phosphatidylserine exposure measured by prothrombinase assay after apoptosis induction in control and Scott cells. Cells were treated for 48 (a) or 72 (b) h, by 200 nM A23187 or 10 μM SKF 96365 in the absence or in the presence of z-VAD.fmk (50 μM), or for 10 min (c) by 1 μM A23187, as described in the "Experimental Procedures". Data are expressed as mean ± SEM of 5 independent experiments (each with duplicate samples), where □ represents the increase in activity with respect to baseline prothrombinase activity. \**P* < 0.05 versus 72 h A23187 treatment of Scott cells. §*P* < 0.05 versus the corresponding value obtained in the absence of z-VAD.fmk. †*P* < 0.05 versus the corresponding value obtained with control cells.



**Figure 7**  
 Phosphatidylserine exposure assessed by Annexin V-labeling after apoptosis induction in control and Scott cells. Cells, treated for 72 h by 200 nM A23187 or 10 μM SKF 96365, were loaded with the fluorochrome Fluo-3 (3 μM) and annexin V-biotin (5 μl at 0.5 mg/ml) and labeling was achieved with phycoerythrin-conjugated streptavidin. Results are expressed as phycoerythrin fluorescence versus cell number. The cell populations with high annexin V labeling correspond to those with high Fluo-3 fluorescence (Fig. 4). One representative experiment out of four performed likewise is shown.

way of Ca<sup>2+</sup> entry should trigger apoptosis in cells with a normal store-operated Ca<sup>2+</sup> entry, as observed in other cells [19,20]. SKF 96365, an inhibitor of store-operated Ca<sup>2+</sup> channels [25], was able to induce apoptosis in both control and Scott cells in the same proportions. These results are consistent with the observations of Jayadev et al. [19] and indicate that after inhibition of store-operated Ca<sup>2+</sup> channels, cells acquire a high propensity to undergo apoptosis. Also, the importance of store-operated Ca<sup>2+</sup> entry in maintaining cell viability has been observed in HL60 cells after induction of apoptosis by SKF 96365 [20]. Here, SKF 96365 did not affect [Ca<sup>2+</sup>]<sub>i</sub> in both cell types, and the data obtained with SKF 525A, an

inhibitor of cytochrome P450 that also blocks store-operated  $\text{Ca}^{2+}$  influx [28], confirm the relationship between store-operated  $\text{Ca}^{2+}$  entry and apoptosis and suggest that, in B cells,  $\text{Ca}^{2+}$  entry is regulated, at least in part, by the cytochrome P450 enzymatic system.

It has been proposed that  $\text{Ca}^{2+}$  release from stores is by itself sufficient to induce apoptosis [34,35]. Preston et al. [36] have shown that decreased  $\text{Ca}^{2+}$  in endoplasmic reticulum stores due to partial reduction of capacitative  $\text{Ca}^{2+}$  entry leads to poor refilling of these stores and to apoptosis. In the present study, combinations of SKF 96365 + A23187 or SKF 525A + A23187 resulted in a weak degree of apoptosis in control cells, which was lower than in cells treated with  $\text{Ca}^{2+}$  entry inhibitors alone (i.e. in the absence of A23187).  $[\text{Ca}^{2+}]_i$  measurements suggest that even when store-operated  $\text{Ca}^{2+}$  entry is inhibited, A23187-induced  $\text{Ca}^{2+}$  influx remains protective against apoptosis in control cells.

Scott cells show a lack of rapid procoagulant phosphatidylserine exposure after drastic ionophore stimulation [11,12,14]. Here, both control and more markedly Scott cells externalized phosphatidylserine after induction of apoptosis, indicating that this process when accounting for the procoagulant response after a swift elevation of intracellular  $\text{Ca}^{2+}$  depends on a different mechanism and/or involve different transporters. This does not rule out that some steps are however common. Our observations are consistent with those reported in Jurkat [37] or Raji cells [38], where it has been suggested that phosphatidylserine exposure is regulated by multiple pathways. Very recently, Williamson et al. [15] have shown that Scott EBV-lymphoblasts from another patient are able to externalize phosphatidylserine during apoptosis. In addition, it is well established that  $\text{Ca}^{2+}$  influx is necessary to promote the migration of phosphatidylserine in the membrane outer leaflet [13]. Then, how phosphatidylserine can be externalized in Scott cells if  $\text{Ca}^{2+}$  entry is reduced? One possible explanation is that a ~30% reduction of  $\text{Ca}^{2+}$  influx is harmless for a normal exposure of phosphatidylserine during nucleated cell apoptosis, but not for the rapid exposure of phosphatidylserine by platelets necessary for the hemostatic response. Phosphatidylserine externalization is believed to result from the inhibition of aminophospholipid translocase and the activation of  $\text{Ca}^{2+}$ -dependent outer transport of phospholipids possibly mediated by a nonspecific phospholipid scramblase [15,39,40] or a vectorial transporter such as ABCA1 [41]. In this respect, phosphatidylserine exposure does not correlate with scramblase expression [38]. Moreover, Scott cells show a normal scramblase expression [42–44] and, although ABCA1 has been reported to participate in the transbilayer movement of phosphati-

dylserine [41], the involvement of other transporters cannot be ruled out [45].

Finally, we have observed a dissociation of membrane changes of cells undergoing apoptosis from other features of programmed death, such as DNA fragmentation and decrease of mitochondrial  $\Delta\psi$ . While the latter was caspase-dependent after 48 h treatment, phosphatidylserine exposure was not. However, after 72 h treatment, DNA fragmentation and changes of mitochondrial  $\Delta\psi$  were caspase-independent in both control and Scott cells, whereas procoagulant phosphatidylserine exposure induced by A23187, during the same time period, was inhibited by the caspase inhibitor z-VAD.fmk in Scott but not in control cells. Nevertheless, a more specific caspase-3 inhibitor had no effect on DNA fragmentation,  $[\text{Ca}^{2+}]_i$  and phosphatidylserine exposure, suggesting that caspase-3 pathway is not involved in these apoptotic features. However, the participation of other caspases cannot be ruled out. The mechanisms controlling membrane phosphatidylserine redistribution during apoptosis are certainly complex, some studies pointing to the importance of caspase activation [5,46,47], whereas in other models, caspase are not involved [48,49]. In B cells particularly, apoptotic pathways seem to be partitioned into caspase-independent and caspase-dependent steps, phosphatidylserine exposure following BCR activation being a caspase-dependent process, but not change of  $\Delta\psi$  [50]. Also, caspase inhibition after BCR stimulation blocks DNA fragmentation but does not prevent cytochrome c release and cell death [51]. The present results are in favor of alternative pathways used by Scott cells in order to allow normal viability, apoptosis and perhaps phagocytosis, which may explain the absence of other apparent pathogenic consequences of the syndrome.

## Conclusions

In summary, inhibition of store-operated  $\text{Ca}^{2+}$  channels induces caspase-independent apoptosis in control and Scott cells. In the latter, externalization of phosphatidylserine during apoptosis is however dissociated from other intracellular changes, suggesting a plastic adaptability of the mechanisms governing phosphatidylserine transmembrane redistribution in cells undergoing specialized stimulation and those entering programmed death.

## Materials and Methods

### Reagents

X-VIVO 15 medium was from BioWhittaker (Walkersville, MD). PI, type I-A RNase A and Phycoerythrin-conjugated streptavidin were from Sigma Chemical Co. (St Louis, MO).  $\text{Ca}^{2+}$  ionophore A23187, SKF 96365, SKF 525A, z-VAD.fmk and Ac-DEVD-CHO were from Calbiochem (La Jolla, CA). Fluo-3/AM and JC-1 were from Mo-



lecular Probes (Eugene, OR). TUNEL assay kit was from Roche Diagnostics (Mannheim, Germany). Biotin-labeled annexin V has been prepared and characterized as previously described [52].

#### **Culture of Scott B lymphoblastoid cells**

The case of Scott syndrome has been detailed in another study where homozygous status has been suggested [12]. Lymphocytes were isolated under sterile conditions and stored in liquid nitrogen. Infection of B lymphocytes by EBV (EBV B958, Marmouset) was performed in RPMI culture medium containing 20% FCS in the presence of 50 ng/ml cyclosporin and was achieved within 3 weeks [12]. Three independent EBV infections could be performed. It should be noted that the Scott phenotype was observed in these cells after drastic activation with  $Ca^{2+}$  ionophore A23187, as previously described [12,22]. EBV-infected B cells were expanded in X-VIVO 15 culture medium (free  $Ca^{2+}$  concentration = 1.8 mM).

#### **Induction of apoptosis**

The cell viability was checked by Trypan blue exclusion. Cells were seeded at  $5 \times 10^5$  cells/ml in the presence or absence of A23187 (100–200 nM) for 48 or 72 h. In some experiments, cells were treated with 10  $\mu$ M SKF 96365 or 10  $\mu$ M SKF 525A in the absence or presence of  $Ca^{2+}$  ionophore A23187 (200 nM) for 48 or 72 h.

In another set of experiments, cells cultured in a standard 6-well plate were submitted to UV light by exposure to the germicidal lamp (peak sensitivity approximately 254 nm) in the tissue culture hood for 10, 20 et 30 s (5, 10 and 15 J/cm<sup>2</sup>, respectively) and apoptosis was measured 18 h after irradiation.

#### **Determination of hypodiploid DNA**

After the different treatments, cells were harvested and numbered. Concentration was adjusted to  $5 \times 10^5$  cells/ml of 70% ethanol solution in H<sub>2</sub>O, and fixation was allowed to proceed during at least 1 h at 4°C. Cells were washed once in HBSS before resuspension in a solution containing type I-A RNase A (0.5 mg/ml) in HBSS and were incubated for 10 min at 37°C. PI was then added at a final concentration of 0.1 mg/ml. Samples were allowed to stand another 15 min in the dark at room temperature before flow cytometry analysis using CELLQuest software (Becton Dickinson, San Jose, CA).

#### **Measurement of $[Ca^{2+}]_i$ and phosphatidylserine exposure by flow cytometry**

Cells ( $10^6$  cells/ml) were loaded with 3  $\mu$ M Fluo-3/AM for 30 min at room temperature. Cells were then washed twice in X-VIVO 15 medium. Then 10  $\mu$ g/ml PI was added in order to determine the proportion of necrotic cells uptaking PI. Fluo-3 and PI fluorescences were recorded

by flow cytometry. Fluo-3 fluorescence was plotted as FL-1 versus PI fluorescence as FL-2, and both were measured in fluorescence arbitrary intensity units.

In another set of experiments, phosphatidylserine externalization was assessed by monitoring annexin V-biotin binding, simultaneously with  $[Ca^{2+}]_i$  measurement in viable cells only, i.e. in those excluding PI. Briefly, cells were loaded with fluo-3 as described above. After 20 min of Fluo-3 loading, 5  $\mu$ l of annexin V-biotin at 0.5 mg/ml was added. After 10 min of incubation in the dark, at room temperature, cells were washed by one centrifugation-resuspension step in X-VIVO 15 medium. Labeling was achieved following another 10 min incubation at room temperature with phycoerythrin-conjugated streptavidin. Cells were washed by a centrifugation-resuspension step with X-VIVO 15.

#### **Terminal deoxynucleotidyl transferase fluorescein-dUTP nick end labeling (TUNEL) assay**

To assess the occurrence of A23187- or SKF 96365-induced apoptosis in B cells, labeling of fragmented DNA was performed by TUNEL assay using a commercially available cell death detection kit according to the manufacturer's instruction. In brief, cells were fixed with 4% paraformaldehyde solution for 20 min at 4°C and then washed with PBS. Endogenous peroxidases were inactivated by 0.3% H<sub>2</sub>O<sub>2</sub> in a 70% methanol solution for 20 min, then cells were washed with PBS and incubated with permeabilization solution (0.1% Triton X-100) for 2 min at 4°C. Apoptotic cells were incubated with 50  $\mu$ l of TUNEL reaction mixture for 80 min at 37°C. After substrate addition, stained cells were analyzed by flow cytometry. Negative control samples for TUNEL staining lacked terminal deoxynucleotidyl transferase.

#### **Determination of mitochondrial membrane potential ( $\Delta\psi$ )**

$\Delta\psi$  was determined by monitoring the decrease in red fluorescence and the increase in green fluorescence by flow cytometry after labeling the cells with JC-1. Cells suspensions were adjusted to a density of  $5 \times 10^5$  cells/ml and incubated in complete medium with JC-1 (1  $\mu$ g/ml) for 15 min at room temperature in the dark. At the end of the incubation period, the cells were washed twice in PBS. For each experiment, A23187 or SKF 96365-treated and control cells were analyzed for both red and green fluorescences after JC-1 labeling. The mean fluorescence ratio was calculated from the mean red and green fluorescence for the entire sample population [53].

#### **Functional detection of procoagulant phosphatidylserine**

Procoagulant phospholipid exposure in apoptotic cells was detected using a human prothrombinase assay in which phosphatidylserine promotes the activation of prothrombin by factor Xa in the presence of factor Va

[54]. Thrombin generated by assembled prothrombinase complex was measured using a chromogenic assay as described elsewhere [12]. EBV-infected B lymphocytes were studied at  $2 \times 10^5$  cells/ml and the ability to externalize procoagulant phosphatidylserine was examined after treatment by A23187 (200 nM) or SKF 96365 (10  $\mu$ M) in the absence or presence of z-VAD.fmk (50  $\mu$ M) during 48 and 72 h, or 10 min only in case of more drastic stimulation by 1  $\mu$ M A23187. The cells were separated from derived membrane microparticles by centrifugation at 12,000 *g* for 1 min before measurement. For each protocol, results were compared with the prothrombinase activity developed in samples from healthy volunteers. Linear absorbance changes were converted to concentration of generated thrombin by reference to a standard curve. Prothrombinase activity was normalized to  $2 \times 10^5$  cells/ml and was expressed as the increase in activity ( $\square$ ) with respect to baseline prothrombinase activity of untreated cells.

#### Statistical analysis

Experiments were performed at different culture stages. Unpaired Student's *t*-test was used for the statistical analysis. A *P* < 0.05 value was considered significant.

#### Abbreviations

Ac-DEVD-CHO, Ac-Asp-Glu-Val-Asp-CHO; BCR, B cell receptor; Fluo-3/AM, fluo-3/acetocymethyl ester; JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolcarbocyanine iodide; PI, propidium iodide; SKF 525A,  $\alpha$ -phenyl- $\alpha$ -propylbenzeneacetic acid 2-[diethylamino]ethyl ester; SKF 96365, 1-[ $\beta$ -[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole; TUNEL, Terminal deoxynucleotidyl transferase fluorescein-dUTP nick end labeling; z-VAD.fmk, z-Val-Ala-Asp.fluoromethyl ketone.

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