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# Ouabain-induced perturbations in intracellular ionic homeostasis regulate death receptor-mediated apoptosis

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## Abstract

Apoptosis is defined by specific morphological and biochemical characteristics including cell shrinkage (termed apoptotic volume decrease), a process that results from the regulation of ion channels and plasma membrane transporter activity. The $Na^+$-$K^+$-ATPase is the predominant pump that controls cell volume and plasma membrane potential in cells and alterations in its function have been suggested to be associated with apoptosis. We report here that the $Na^+$-$K^+$-ATPase inhibitor ouabain, potentiates apoptosis in the human lymphoma Jurkat cells exposed to Fas ligand (FasL) or Tumor necrosis factor--related apoptosis-inducing ligand (TRAIL) but not other apoptotic agents such as $H_2O_2$, thapsigargin or UV-C implicating a role for the $Na^+$-$K^+$-ATPase in death receptor-induced apoptosis. Interestingly, ouabain also potentiated perturbations in cell $Ca^{2+}$ homeostasis only in conjunction with the apoptotic inducer FasL but not TRAIL. Ouabain did not affect alterations in the intracellular $Ca^{2+}$ levels in response to $H_2O_2$, thapsigargin or UV-C. FasL-induced alterations in $Ca^{2+}$ were not abolished in $Ca^{2+}$-free medium but incubation of cells with BAPTA-AM inhibited both $Ca^{2+}$ perturbations and the ouabain-induced potentiation of FasL-induced apoptosis. Our data suggest that the impairment of the $Na^+$-$K^+$-ATPase activity during apoptosis is linked to perturbations in cell $Ca^{2+}$ homeostasis that modulate apoptosis induced by the activation of Fas by FasL.

### Keywords

Ouabain; calcium; $Na^+$-pump; $Na^+$-$K^+$-ATPase; apoptosis; Jurkat; cell volume; cell shrinkage; AVD; apoptotic volume decrease; extrinsic pathway; Fas ligand; TRAIL; CD95; Apo-1

## INTRODUCTION

Apoptosis is a metabolically active process with importance in organ development and in the regulation and maintenance of cell turnover in various tissues during physiological as well as pathological conditions [1]. This form of programmed cell death is characterized by specific morphological features including cell shrinkage, chromatin condensation, and formation of apoptotic bodies [2]. The signaling cascades that regulate the progression of apoptosis have been extensively studied and characterized. In general both extrinsic and

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intrinsic pathways have been described for the activation of apoptosis. Induction of apoptosis via the extrinsic pathway is triggered by the activation of death receptors such as those activated by Fas ligand or FasL (Fas (CD95/Apo-1) and by the TNF-related apoptosis-inducing ligand or TRAIL (DR4, DR5). Activation of CD95, DR4 and DR5 leads primarily to the formation of the death-inducing signaling complex (DISC) formed by the recruitment of the (Fas-associated death domain (FADD), caspases 8 (and in some cases caspase 10) and the cellular FLICE-inhibitory protein (FLIP). Initiator caspase 8 is processed and activated and further amplifies the apoptotic cascade by activation of executioner caspases. When upon death receptor activation, cells have lower levels of DISC formation and active caspase-8 (Type II cells), the progression of the cell death program relies on an amplification loop induced by cleavage of the Bcl-2-family protein Bid by caspase 8 and its translocation to the mitochondrial to activate the intrinsic pathway of apoptosis [3, 4].

The intrinsic pathway of apoptosis is also referred to as the mitochondrial pathway. It is activated by a wide variety of stimuli including chemotheraputic and cytotoxic agents, stress (radiation, hyperglycemia, hypoxia, oxidative and osmotic stress) and cytokine withdrawal. Although the mechanisms by which these stimuli trigger apoptosis seems to differ between them, they all convey in the release of proapoptotic proteins from the mitochondria including Cyt C. Cytochrome C release induces the formation of the apoptosome, activation of caspase 9 and the further activation of the execution caspases [5, 6].

During apoptosis, loss of cell volume (apoptotic volume decrease, AVD) has been described as a process that facilitates the breakdown of the cell into smaller apoptotic bodies thus aiding their engulfment and consequently phagocytosis by macrophages [7]. We and others have shown that changes in intracellular ionic homeostasis are associated with the loss of cell volume and that these changes play a critical role in regulating the activity of nucleases and caspases during cell death [8-12]. However, the exact mechanisms by which changes in ionic homeostasis regulate apoptosis remains unclear.

When changes in cell volume occur, they normally result from ionic fluxes across the plasma membrane [13], therefore understanding the molecular basis of these ion changes during apoptosis may aid in our understanding of the cell death process. In general, the cells utilize large amounts of ATP to maintain ionic homeostasis required for normal life. In this respect, the $Na^+$-$K^+$-ATPase ($Na^+$-pump) by consuming a considerable proportion of cellular ATP maintains a high $K^+$ and a low $Na^+$ concentration inside the cell, ensuring osmotic balance, cell volume, cytoplasmic pH, ionic gradients and $Na^+$-coupled transport of nutrients and amino acids into the cells [12-14]. To date there is a excellent understanding of the $Na^+$-pump's structure, function and regulation in living cells [14, 15]. Ionic gradient imbalance, particularly those of $Na^+$ and $K^+$, is an early hallmark of initial stages of the apoptotic program. Several reports have associated this phenomenon to a reduction in the $Na^+$-$K^+$ ATPase activity during apoptosis [16-25]. Accordingly, inhibition of the $Na^+$-$K^+$ ATPase with cardiac glycosides, has been widely reported to induce cell toxicity and cell death [21, 26-37], or to enhance apoptosis induced by different stimuli in various model systems [17, 18, 38-40]. However, the molecular mechanisms involved in the regulation of apoptosis by impairment of the $Na^+$-$K^+$-ATPase are still unclear.

In the present study, we report that impairment of the $Na^+$-$K^+$-ATPase activity by ouabain potentiates apoptosis in Jurkat cells upon exposure to Fas ligand (FasL) and Tumor necrosis (TNF)-related apoptosis-inducing ligand (TRAIL), but not by other agents that are known to stimulate primarily the intrinsic mitochondrial apoptotic pathway. Additionally we observed that apoptosis by Fas activation and its potentiation by ouabain are associated with $Ca^{2+}$ release from intracellular stores.

# MATERIALS AND METHODS

## Cell culture

The Jurkat cell clone J6 (human lymphoma) (empty vector transfected, SFFV-*neo*), was a kind gift from Dr., Gerald M. Cohen (Medical Research Council Toxicology Unit, University of Leicester, Leicester, United Kingdom) [41] and was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2mM glutamine, 31mg/l penicillin, and 50mg/l streptomycin and 0.4mg/ml G418 (GIBCO/Invitrogen Co., Carlsbad, CA) at 37°C, 7% $CO_2$ atmosphere. Jurkat cells (5-7 × $10^5$ cells/ml) were incubated with distinct apoptotic inducers for both the extrinsic (FasL or TRAIL [Kamiya Biomedical, Seattle, WA]) and intrinsic (hydrogen peroxide [$H_2O_2$], thapsigargin [Sigma-Aldrich, St. Louis, MO] and 30mJ/$cm^2$ UV-C light [Stratalinker 1800, Stratagene]) pathways of apoptosis over a period of 4h. When indicated, ouabain (Sigma-Aldrich) was added at the concentration indicated at the same time as the apoptotic stimuli and was present throughout the experiment.

Nominally $Ca^{2+}$-free medium was prepared from powdered RPMI (Hyclone) without $Ca^{2+}$ nitrate, supplemented as described above, and with addition of 0.5mM ethylene glycol tetraacetic acid, EGTA (Sigma-Aldrich). Intracellular $Ca^{2+}$ was scavenged by incubation of the cells for 20min with the membrane permeant 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester, BAPTA-AM (25μM; Molecular Probes, Eugene, OR) prior to addition of ouabain and each of the apoptotic stimuli.

## Measurement of cell size by flow cytometry

Light scattered in the forward direction is proportional to cell size, while light scattered at a 90° angle (side scatter) is proportional to cell density or granularity [42]. Cell size and changes in the light scattering properties of the cells were determined by flow cytometry as described previously [43] using a BD LSR II flow cytometer equipped with the BD FACSDiva Software (Becton Dickinson, San Jose, CA) for data analysis. For each sample, 10,000 cells were examined after being excited with a 488nm argon laser while their distribution was recorded on a forward-scatter versus side-scatter plot.

## Measurement of changes in intracellular $Na^+$, $K^+$ and $Ca^{2+}$ by flow cytometry

Changes in the intracellular ionic homeostasis were determined as follows. Jurkat cells were preloaded with either SBFI-AM or CoroNa Green (5μM, 1h) for $Na^+$; PBFI-AM (5μM, 1h) for $K^+$; and Fluo-3 AM (1μM; 30min) for $Ca^{2+}$ (Molecular Probes, Eugene, OR) prior to the end of the incubation period needed to induce apoptosis. Fluorophores were diluted in dimethyl sulfoxide (DMSO) with a final concentration of 0.1%. Ten thousand cells were examined by sequential excitation of the cells containing PBFI, SBFI (340-350nm) or Fluo-3 (488nm) and examining their emissions at 425nm (PBFI/SBFI) or 530nm (Fluo-3).

## Measurement of changes in plasma membrane potential

Changes in the plasma membrane potential were measured by flow cytometry using DiBAC4(3) (Molecular Probes). DiBAC4(3) was prepared in DMSO (0.1% final concentration). Thirty minutes prior to each time of examination, DiBAC4(3) was added to a final concentration of 150ng/ml. Ten thousand cells were examined under each condition with excitation performed using a 488nm argon laser, and fluorescent emission was detected at 530nm.

### Changes in Plasma Membrane Integrity

To evaluate plasma membrane integrity, propidium iodide (PI) (10μg/ml; Sigma-Aldrich) was added to the cells prior to flow cytometry analysis. PI uptake was evaluated by its excitation with an Argon 488nm laser whereas emission was acquired with a 695/40 filter. High PI fluorescence indicates a loss of membrane integrity.

### Analysis of degraded DNA content by flow cytometry

Samples were pelleted and washed in ice-cold PBS. Then, cells were fixed by the addition of 70% cold ethanol to a volume of 1.5ml, adjusted to 5ml with cold 70% ethanol, and stored at -20 °C overnight. Fixed cells were pelleted, washed once in PBS and stained in 1 ml of 20μg/ml PI, 1mg/ml RNase, in PBS for 20 min at RT. Seven thousand five hundred cells were analyzed by flow cytometry using a Becton Dickinson FACSort flow cytometer, and BD Cell Quest Software (Becton Dickinson) for analysis. Region gates were set on a PI area versus width dot plot to exclude cell debris and cell aggregates.

### Determination of caspase 3/7-like activity by flow cytometry

Caspase 3/7-like activity was determined using CaspaTag *In Situ* Apoptosis Detection Kit (Millipore, Billerica MA). Cells were incubated for 1h prior to analysis at 37°C, 7% $CO_2$ atmosphere with FLICA reagent (10μM FAM-DEVD-FMK)), a cell permeable caspase 3/7-like substrate that fluoresces upon cleavage at the DEVD site. Ten thousand cells were excited at 488nm and examined at 530nm for the fluorescence intensity of the FLICA substrate.

### Statistical analysis

All calculations were performed using the JMP software (SAS Institute, Cary, NC). Means were compared by one-way analysis of variance followed by a Student's t-test. A value of $P < 0.05$ was considered significant.

## RESULTS

### Death receptor-induced apoptosis is potentiated in the presence of ouabain

We used a flow cytometric-based approach to assess apoptotic volume decrease or cell shrinkage. As a cell shrinks, there is a decrease in the amount of forward-scattered light that is proportional to cell size in coordination with a change in side-scattered light that is relative to cell density or granularity. Figure 1A shows a reduction in the amount of forward-scattered light at each concentration of FasL in the absence (- Ouabain) or presence of ouabain (100μM) (cells outside of the gated area assigned for normal cells). Cell shrinkage or AVD was directly proportional to the concentration of FasL. The addition of ouabain increased the number of apoptotic cells for each concentration of FasL tested (Fig. 1A, Table 1). We next evaluated if ouabain enhanced the occurrence of other parameters of apoptosis. Figure 1B shows that the percentage of cells with degraded DNA content was also enhanced in the presence of ouabain for any given concentration of FasL. Similarly, Figure 1C shows that the percent of FLICA-positive cells indicating active caspase 3/7 like activity was also significantly higher in ouabain-exposed cells when treated with 50 and 100ng/ml FasL. Together, these data demonstrate that impairment of the $Na^+$-$K^+$-ATPase by ouabain potentiates FasL-induced apoptosis, as determined by AVD, DNA degradation and execution caspase activity.

Ouabain is a specific inhibitor of the $Na^+$-pump, which results in a depletion of intracellular $K^+$ and the accumulation of intracellular $Na^+$. Figure 2A shows the change in intracellular $K^+$ and $Na^+$ upon treatment with 100 uM ouabain, 50 ng/ml FasL, or both, using ionic

fluorescent indicators. We have used two independent $Na^+$ indicators due to the low selectivity of $Na^+$ fluorescent dyes. Previously, we demonstrated that FasL treated Jurkat cells results in an increase in intracellular $Na^+$, prior to the loss of both intracellular $Na^+$ and $K^+$ [18, 44]. Here we observed a similar pattern in response to FasL using both the SBFI and CoroNa Green dyes (Figure 2A). As expected, ouabain alone induced a decrease in intracellular $K^+$, along with an increase in intracellular $Na^+$. However, the combined treatment of FasL and ouabain resulted in a greater increase in intracellular $Na^+$ compared to either FasL or ouabain alone. Interestingly, the late stage of both intracellular $Na^+$ and $K^+$ loss was not observed. Additionally, we used the PBFI and CoroNa Green ionic indicators in combination to show that the initial increase in intracellular $Na^+$ occurs prior to the dramatic loss of both $Na^+$ and $K^+$ (Figure 2B).

Recently, cell death has been reported to be induced by ouabain independent of alterations in the ionic gradients of $Na^+$ and $K^+$ [45-47]. In Jurkat cells, apoptosis may be induced using concentrations of ouabain 20 times lower than those needed to inhibit the pump [48] suggesting that mechanisms other than ionic transport inhibition may be operative. Potentiation of the death receptor pathway of apoptosis, using lower concentrations of ouabain has also been described in activated lymphocytes [40]. We next decided to determine if ouabain might be activating a $Na^+$/ $K^+$-independent signaling cascade regulating apoptosis. For this we used low concentrations of ouabain where no alteration in ionic gradients is observed. Figure 2C shows that concentrations of 100nm or less of ouabain did not significantly alter the ionic gradients in Jurkat cells reflected by the absence of plasma membrane depolarization. At the same concentrations, ouabain was not able to potentiate FasL-induced apoptosis (Figure 2D). These results suggest that the effects of ouabain on apoptosis are associated to its ability to inhibit the $Na^+$-$K^+$-ATPase and alter cellular ionic homeostasis.

To determine if ouabain-induced potentiation of apoptosis was selective for FasL-induced cell death, we evaluated the effect of ouabain on apoptosis induced by other apoptotic stimuli. Figure 3A shows that the presence of ouabain did not result in a significant increase in apoptosis assessed by the occurrence of cell shrinkage after exposure to either $H_2O_2$ (500μM), thapsigargin (5μM) or UV-C (30mJ/cm$^2$) as compared to FasL treatment. However, the presence of ouabain significantly increased the percentage of shrunken cells after exposure to TRAIL when compared to control conditions (Fig. 3B, Table 2) in a manner analogous to FasL induced apoptosis. Together these data suggest that the effect of ouabain in potentiating apoptosis is largely selective to the extrinsic apoptotic pathway activated by the death receptors for FasL and TRAIL, but not to the intrinsic or mitochondrial pathway(s) of apoptosis.

## Ouabain-induced potentiation of FasL-induced apoptosis involves perturbations in cell $Ca^{2+}$ homeostasis

Efficient $Na^+$-$K^+$-ATPAse activity has been shown to be necessary for the maintenance of not only $Na^+$ and $K^+$ homeostasis, but also other cellular ionic gradients including $Ca^{2+}$ [14]. Thus, we hypothesized that disruption of the ionic homeostasis by ouabain might also result in a secondary alteration in intracellular $Ca^{2+}$ which is known to regulate several components of apoptosis. Figure 4A shows that FasL induced cell shrinkage was paralleled by an increase in intracellular $Ca^{2+}$ content as shown by changes in Fluo-3 fluorescence. Additionally, this increase in intracellular $Ca^{2+}$ by FasL was enhanced in the presence of ouabain. Histograms of the number of cells with an increase in $Ca^{2+}$ show that FasL induced a dose-dependent increase in intracellular $Ca^{2+}$, which was significantly higher in the presence of ouabain (Fig. 4B and 4C)

Exposure of cells to ouabain did not result in a significant increase in the number cells with a high intracellular $Ca^{2+}$ concentration induced in the presence of $H_2O_2$ (500μM), thapsigargin (5μM), or UV-C (30mJ/cm$^2$) (Fig. 5, Table 3). Interestingly, although there was a small increase in the intracellular $Ca^{2+}$ concentration induced by TRAIL (500ng/ml) upon ouabain treatment, this was not statistically significant when averaged (Figure 5, Table 3). Together, these data strongly suggest a specific link between the impairment of the $Na^+$-$K^+$-ATPase activity and changes in the free intracellular $Ca^{2+}$ during FasL-induced apoptosis but not other apoptotic stimuli. It has been widely reported that thapsigargin rapidly induces a transient $Ca^{2+}$ release from the endoplasmic reticulum that is immediately counteracted by the activity of the plasma membrane $Ca^{2+}$ pump or the $Na^+/Ca^{2+}$ exchanger (NCX). This explains why cells treated with thapsigargin after four hours do not show a marked increase in $Ca^{2+}$ content.

### Ouabain-induced potentiation of FasL-induced apoptosis is associated to changes in cell $Ca^{2+}$ homeostasis that results from the impairment of the $Na^+$-$K^+$-ATPase activity

Our results suggest a specific link between the impairment of the $Na^+$-$K^+$-ATPase activity and changes in the intracellular $Ca^{2+}$ homeostasis during FasL-induced apoptosis. To further understand this link, we next determined the source for $Ca^{2+}$ overload during FasL-induced apoptosis and its potentiation by ouabain. Figure 6A shows that nominally $Ca^{2+}$-free medium did not prevent FasL-induced intracellular $Ca^{2+}$ release. These data suggest that extracellular sources do not significantly contribute to the elevation in intracellular $Ca^{2+}$ content in response to FasL and ouabain. In contrast, preloading cells with 25μM BAPTA-AM, a cell permeable $Ca^{2+}$ scavenger that is cleaved by cytoplasmic esterases and consequently buffers free $Ca^{2+}$, completely abolished the rise in intracellular $Ca^{2+}$ content observed during FasL stimulation either in the presence or absence of ouabain (Fig. 6B).

To elucidate the role of intracellular $Ca^{2+}$ in FasL-induced apoptosis and on its potentiation by ouabain, cells were pre-incubated for 20min with 25μM BAPTA-AM. The presence of BAPTA-AM significantly reduced apoptosis observed in the presence or absence of ouabain (Fig. 7, Table 4). Moreover, the percentage of apoptotic cells in the presence of BAPTA-AM and BAPTA-AM plus ouabain was not significantly different suggesting that ouabain induced potentiation of apoptosis is mediated by stimulation of intracellular $Ca^{2+}$ overload. Additionally, BAPTA-AM also significantly reduced the percentage of cells with degraded DNA induced by Fas activation and potentiated by ouabain (not shown). These data suggest that FasL-induced apoptosis and its potentiation by ouabain involve a rise in intracellular $Ca^{2+}$. Thus, the regulation of FasL-induced apoptosis by the $Na^+$-$K^+$-ATPase activity seems to be specifically regulated by alterations in intracellular $Ca^{2+}$ homeostasis.

## DISCUSSION

Apoptosis is a physiological mode of cell death by which unwanted cells are removed in response to selective stimuli. This mode of cell death has been the subject of extensive research because of its involvement in human diseases [1]. Cell death by apoptosis is a process well characterized and defined by many distinct morphological and biochemical characteristics. Among these parameters is cell shrinkage or AVD that is independent of cell type and stimulus employed to induce cell death. AVD has been shown to be driven by the movement of ions across membranes. Such alterations in ionic homeostasis are important because they have also been shown to modify many biological processes including metabolism and gene expression [7, 10, 11, 13].

We and others have previously demonstrated that apoptosis can impair the activity of the $Na^+$-$K^+$-ATPase [17, 18, 21, 22, 49, 50]. The present study now examined the role of the $Na^+$-$K^+$-ATPase with various apoptotic stimuli by its inhibition with ouabain. Ouabain

potentiation of apoptosis in Jurkat cells was restricted to the death receptor pathway (FasL and TRAIL) since it had no effect when other stimuli ($H_2O_2$, thapsigargin and UV-C) were used to activate the cell death process. Although these results are in agreement with other studies showing that ouabain and other cardiac glycosides potentiate apoptosis [17, 18, 38-40], our data demonstrates for the first time that the effect of ouabain is restricted to the extrinsic death receptor pathway in lymphoid cells. Additionally, we showed that in FasL-induced apoptosis, impairment of the $Na^+$-$K^+$-ATPase results in an increase in $Ca^{2+}$ release from intracellular stores that specifically regulates the progression of the cell death program. Recent reports have shown that degradation of the $Na^+$-$K^+$ ATPase occurs during apoptosis induced by death receptor activation [18] glucocorticoids [25] and staurosporine [51]. On the other hand apoptotic resistant phenotypes of bcl-2 overexpressing cells [52, 53] have been associated with an elevated $Na^+$-$K^+$ ATPase activity. However, the mechanisms by which apoptosis modulates ATPase activity are still unclear. Recent reports suggest the role of caspases [25, 51], protein kinases [24, 54], and reactive oxygen/nitrogen species [22, 50, 55-57] on the modulation of $Na^+$-$K^+$-ATPase expression and activity during apoptosis.

The $Na^+$-$K^+$-ATPase exports three $Na^+$ ions and imports two $K^+$ ions (at the expense of one ATP molecule) and thus serves to maintain high $K^+$ and low $Na^+$ intracellular concentrations. Failure or inhibition of the pump results in intracellular depletion of $K^+$ and consequently accumulation of $Na^+$ both of which lead to plasma membrane depolarization [12, 14, 58]. Because the $Na^+$ and $K^+$ gradients maintained by the $Na^+$-$K^+$-ATPase have been shown necessary for the maintenance of the overall ionic homeostasis of the cell [12, 14, 15, 58], we analyzed the relationship between the activity of the $Na^+$-$K^+$-ATPase and changes in intracellular $Ca^{2+}$ levels. Interestingly, ouabain only potentiated intracellular $Ca^{2+}$ release induced by FasL but not by other apoptotic stimuli. This result was surprising when we take into account that ouabain potentiated cell shrinkage or AVD in Jurkat cells treated with TRAIL. Furthermore, it has been stipulated that activation of the Fas receptor and DR4 and DR5 (TRAIL receptors) lead to common signaling apoptotic pathways including the formation of DISC formed by the recruitment of the FADD) caspases 8 and FLIP. Previous reports have suggested that FasL- and TRAIL-induced apoptosis might be distinctively regulated by Bcl-2 proteins [59-61]. Thus, it is possible that although ouabain potentiates apoptosis and AVD induced by activation of death receptors for TRAIL and FasL, it does this by distinct signaling events.

The data described above suggest that ouabain has a specific effect on FasL-induced apoptosis through the regulation of changes in intracellular $Ca^{2+}$ content. We observed that the presence of $Ca^{2+}$-free medium did not abolish the rise in intracellular $Ca^{2+}$ induced by FasL. In contrast, the intracellular $Ca^{2+}$ scavenger BAPTA-AM prevented FasL-induced $Ca^{2+}$ increase, which demonstrates that $Ca^{2+}$ release from intracellular stores rather than its influx from the extracellular medium are involved in the $Ca^{2+}$ overload during apoptosis induced by activation of Fas and its potentiation by ouabain. This finding also argues against the possibility that the intracellular $Ca^{2+}$ rise might result from the reversal of the NCX (where intracellular $Na^+$ is exchanged for extracellular $Ca^{2+}$) since it would require extracellular $Ca^{2+}$ [62]. However, it is important to mention that inhibition of the $Na^+$-$K^+$-ATPse by ouabain might lead to the impairment of NCX activity by inversion of the $Na^+/K^+$ gradient. This might in turn contribute to further accumulation of released $Ca^{2+}$ because the NCX is one of the main pathways for intracellular $Ca^{2+}$ clearance [63]. It has been widely reported that the effect of cardiac glycosides on $Ca^{2+}$ signaling and overload is linked to the activity of NCX [64-71].

Calcium is known to regulate both extrinsic and intrinsic pathways of apoptosis at the level of the mitochondria [72]. Jurkat cells are considered type II cells which means that the activation of death receptors leads to a limited amount of DISC formation and activation of

caspase 8. Thus type II cells depend on a positive feedback loop triggered by the release of mitochondrial cytochrome c and the activation of caspase-9 for the efficient execution of the cell death program [73]. Thus, the regulatory role of ouabain on FasL-induced $Ca^{2+}$ perturbations might occur prior to the activation of the mitochondrial pathway of apoptosis. We and others have previously shown that changes in intracellular $Ca^{2+}$ are necessary for DNA degradation during late stages of apoptosis induced by activation of Fas [74, 75]. Recently, Wozniak et al., reported that apoptosis induced by FasL is regulated by early oscillatory $Ca^{2+}$ signals prior to the release of cytochrome C [76]. In this work, the authors demonstrated that Fas-induced apoptosis required an early endoplasmic reticulum (ER)-mediated $Ca^{2+}$ release induced by phospholipase C-gamma1 (PLC-gamma1) phosphorylation and $Ca^{2+}$ release from inositol 1,4,5-trisphosphate receptor (IP3R) channels [76]. Indeed previous reports have demonstrated that T cells deficient in IP3R are resistant to Fas-induced apoptosis [77]. The mechanism by which Fas activation mediates PLC-induced $Ca^{2+}$ release is still unknown, however, it has been previously reported that T-cells deficient of FADD have impaired $Ca^{2+}$ release from intracellular stores [78].

Similarly to Fas, ouabain has been reported to induce $Ca^{2+}$ oscillation by a direct interaction of the $Na^+$-$K^+$-ATPase with the IP3R [66, 79, 80]. Moreover, ouabain has also been shown to induce the Src-dependent phosphorylation of PLC-gamma1 [81] and induce/potentiate $Ca^{2+}$ release from the ER. We report here that elimination of ouabain-induced $Ca^{2+}$ overload from intracellular stores through the use of BAPTA-AM abolishes the potentiation of FasL-induced apoptosis observed via direct inhibition of the $Na^+$-$K^+$-ATPase using ouabain. Thus it is plausible that ouabain might be regulating apoptosis upon Fas stimulation by acting on this early PLC-mediated $Ca^{2+}$ release from the ER prior to the activation of the mitochondria feedback loop, which will explain why ouabain does not potentiate apoptosis induced by intrinsic pathways. In fact, a recent report demonstrates that apoptosis induced by activation of the mitochondrial (intrinsic) pathway does not depend on $Ca^{2+}$ release IP3R receptors [82].

Ouabain and other $Na^+$-$K^+$-ATPase inhibitors seem to have tissue-specific effects on the modulation of cell death (by either necrosis or apoptosis) and cell survival. Inhibition of the $Na^+$-$K^+$-ATPase with cardiac glycosides, has been widely reported to induce or enhance apoptosis induced in various model systems including lymphocytes [17, 18, 30, 40], neural cells [27, 34, 37, 39] and a variety of other cell types [21, 26, 28, 29, 32, 33, 38]. In contrast, other studies have reported that chronic inhibition of the $Na^+$-$K^+$-ATPase activity does not affect survival of vascular smooth muscle cells (VSMC) [83, 84], lymphocytes [85, 86] and astrocytes [87]. We have previously reported the potentiation of Fas-induced apoptosis in Jurkat cells by ouabain [18]. Previous studies have demonstrated that chronic inhibition of the $Na^+$-$K^+$-ATPase with nM concentrations of cardiac glycosides induces apoptosis in Jurkat cells [48, 88]. Interestingly, contradictory results have been reported by Orlov and collaborators [84]. In this study, Orlov et al. described that Jurkat cells preincubated 2h with high concentrations of ouabain (1mM) did not observe an increase in apoptosis after stimulation with FasL [84]. In contrast, in our studies, ouabain was added in parallel to the induction of apoptosis. Pretreatment of ouabain seems to protect against apoptosis induced by different stimuli [83, 84, 89-92]. It is likely that prolonged alterations in the intracellular ionic homeostasis lead to altered conditions for apoptosis signaling. In fact it is known that prolonged ouabain treatment triggers the activation of PKC-, PI3K-, MAPK-survival pathways and the induction up-regulation of anti-apoptotic proteins such as Bcl-2 and mortalin [86, 89-95], which might occur independently from alterations in the $Na^+$ gradient [15, 96]. We hypothesize then that activation of these signaling cascades alters the cellular environment prior to the induction of apoptosis impairing death receptor signaling. These observations might explain these contradictory observations.

Recently, it was demonstrated that ouabain binding to the $Na^+$-$K^+$-ATPase induces cell signaling events independent from $Na^+$ gradient/transport [45, 65, 66, 97]. Orlov et al., demonstrated that ouabain binding to the $Na^+$-$K^+$-ATPase induces cell death by $Na^+/K^+$-independent activation of p38 MAPK. In this work, however, cells were exposed to chronic incubations with ouabain which were shown to induce necrosis rather than apoptosis [98]. Non-apoptotic cell death is also induced by chronic treatment of MDCK cells [47, 99] and porcine aorta endothelial cells [88]. These results clearly indicate that ouabain might also regulate other distinct types of cell death by different signaling pathways in a variety of cell types which might depend as well on the incubation time of the cardiac glycoside.

Ihenetu and coworkers recently demonstrated that chronic inhibition of the $Na^+$-$K^+$-ATPase with cardiac glycosides, at concentrations 20 times lower than that needed to inhibit the ATPase induces apoptosis in Jurkat cells [48]. These results suggested that mechanisms other than ionic transport inhibition may be operative. However, in this study, the IC50 required for inhibition of $Na^+$-$K^+$-ATPase was determined in porcine cerebral cortex tissue but not in Jurkat cells. In fact, Nobel and coworkers previously determined that at concentrations below 0.5μM ouabain does not inhibit the $Na^+$-$K^+$-ATPase activity measured by the ability of the cells to take up radiolabeled $^{86}Rb^+$ as a tracer for $K^+$, instead, ouabain was reported to stimulate the activity of the $Na^+$-pump [17]. This phenomenon has been reported in other cell types [100]. Similar to these results, in this work we have demonstrated that ouabain, at concentrations that do not induce any alteration in the ionic gradients, reflected by the lack of plasma membrane depolarization, had no effect on AVD or apoptosis.

As reviewed elsewhere [13], cells contain impermeable, polyvalent anionic macromolecules and are constantly threatened by colloid osmotic cell swelling due to the entrance of diffusible ions and water. According to the “pump and leak” concept, cell swelling is avoided by low $Na^+$ permeability and active $Na^+$ extrusion via the $Na^+$-$K^+$-ATPase which renders the plasma membrane impermeable to $Na^+$. The $Na^+$-K+-ATPase builds up the outwardly directed gradient for $K^+$ and in combination with the high $K^+$ permeability of the plasma membrane, generates an inside negative membrane potential (Em), which drives $Cl^-$ out of the cell, allowing essential organic molecules within the cytoplasm [101-104]. It is expected then, that inhibition of the $Na^+$-$K^+$-ATPase by ouabain will induce cell swelling in Jurkat cells as it has been reported in other cell types where ouabain induces cell death by necrosis concomitant with cell swelling [47, 88, 99, 105] (see above). After pump inhibition, the fall in the $K^+$ gradient should collapse the Em, which would result in the influx of $Cl^-$ and $Na^+$ leading to cell swelling. However, in Jurkat cells, ouabain by itself does not induce apoptosis or cell shrinkage/AVD. AVD has been shown to be mediated by the activation of volume regulated $K^+$ and $Cl^-$ channels which should counteract influx of $Cl^-$ triggered by inhibition of the $Na^+$-$K^+$-ATPase [8, 106-109]. We have previously demonstrated that during apoptosis, intracellular $Na^+$ increase (mediated by impairment of the $Na^+$-$K^+$-ATPase activity and activation of $Na^+$ channels) is followed by a secondary loss of intracellular $Na^+$ which counteracts $Na^+$ overload [8, 18, 44]. Although it is possible that the absence of cell swelling in ouabain treated Jurkat cells is related to tissue specific effects of ouabain, it is more likely that cell swelling is counteracted by the AVD process induced by either FasL (when ouabain treatment is done acutely) or by chronic treatment with ouabain. In fact, it has been previously demonstrated that in those cell types where ouabain induces/potentiates apoptosis, treatment with the cardiac glycoside triggers a transient cell swelling followed by AVD and cell shrinkage [37, 39].

We have recently demonstrated that AVD occurs in two stages characterized by an initial reduction in cell volume associated with the influx of $Na^+$ ions followed by a secondary shrinkage linked to the loss of both $K^+$ and $Na^+$ [8, 18, 44]. Cell shrinkage is a hallmark of

apoptotic cell death, but it is as yet unclear whether a reduction in cell volume is a primary signal for the activation of apoptosis or just a consequence of the alterations in ionic gradients [110-112]. We have previously demonstrated that sodium influx but not cell shrinkage or AVD is required for the progression of apoptosis [44]. In our study ouabain treatment reduced $K^+$ content. However, ouabain-induced $K^+$ loss was not sufficient to induce apoptosis. This result might be explained by the fact that under the experimental conditions tested here (4h incubation with ouabain), ouabain does not decrease $K^+$ to the same extent as it occurs during apoptosis triggered by stimuli such as FasL (Figure 2). Indeed, we have observed that chronic treatment of Jurkat cells for18h with 100μM ouabain induces caspase-dependent apoptosis (Razik and Cidlowski data not shown).

In conclusion, we report here that ouabain selectively potentiates apoptosis in Jurkat cells induced by activation of death receptors which suggests a role for the $Na^+$-$K^+$-ATPase during apoptosis. Interesintgly, our results also suggest that apoptosis by Fas activation and its potentiation by ouabain are linked to $Ca^{2+}$ release from intracellular stores. In contrast, the regulation of TRAIL-induced apoptotis and AVD by ouabain was shown to involve a different mechanism independent from alterations in intracellular $Ca^{2+}$. Our data demonstrates a link between the impairment of the $Na^+$-$K^+$-ATPase during apoptosis and changes in cell $Ca^{2+}$ homeostasis that are exclusively related to the FasL-induced apoptosis.

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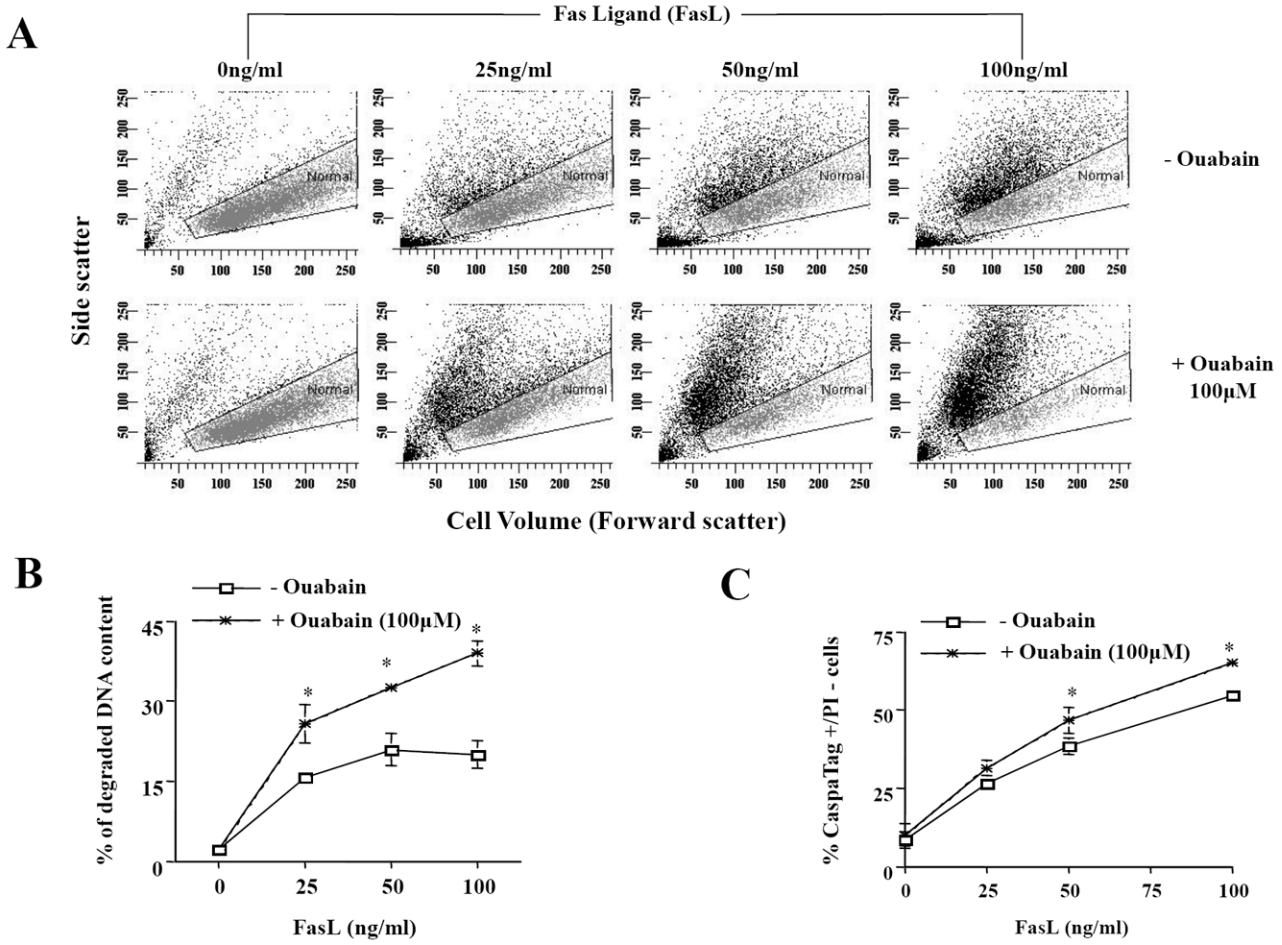


**Fig. 1. Effect of ouabain on FasL-induced apoptosis**

Apoptosis was induced by addition of FasL for 4h in the presence or absence of ouabain at a final concentration of 100μM. Cells were analyzed for changes in cell size on a forward- versus side-scatter plot **(A)**, DNA content **(B)**, and caspase 3/7-like activity **(C)** as explained in materials and methods. In **(A)**, a gate was drawn to depict cells with normal cell size *(grey dots)*. An increase in the number of apoptotic cells (shrunken cells) is reflected as an increasing number of cells falling outside the gated area assigned for normal cells. Cell shrinkage or AVD is observed as a decrease in the amount of forward-scattered light which is proportional to cell size in coordination with a change in side-scattered light that is proportional to cell density or granularity. In **B**, data represents the % of cells with subdiploid DNA content In **C**, data represent the % of cells with increased FLICA (increased activation of execution caspases) and low PI fluorescence (integral plasma membrane integrity). Shown are either averaged values ± SEM from three independent experiments **(B** and **C)**, or a single representative experiment from five independent experiments **(A)**. Asterisks (*) indicate statistical significance ($P<0.05$) between the two experimental conditions at each concentration of FasL.

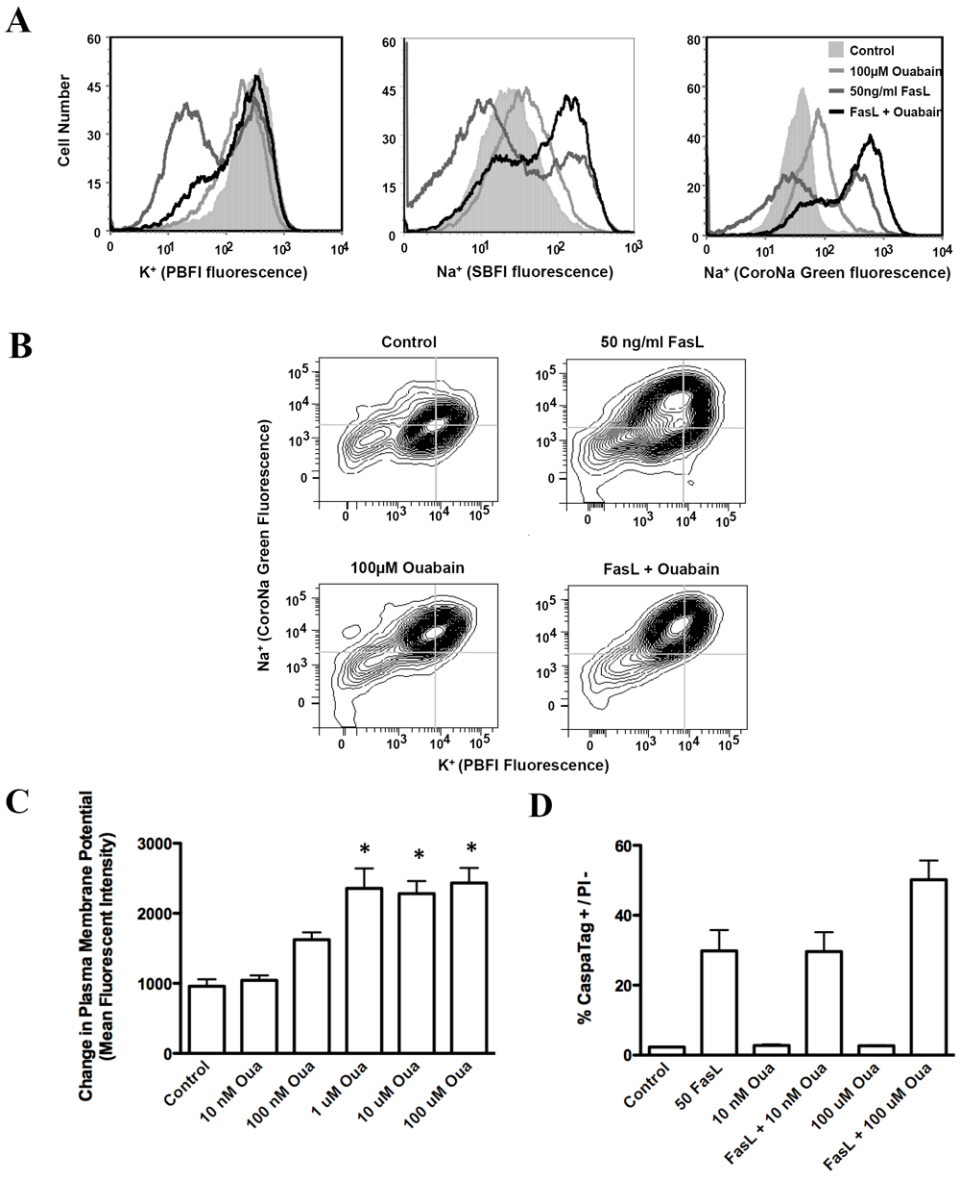


**Fig. 2. Effect of ouabain on intracellular $K^+$ and $Na^+$ content**

Apoptosis was induced by addition of FasL for 4h in the presence or absence of ouabain. For analysis of $K^+$ and $Na^+$ content, one hour prior to examination, SBFI-AM, CoroNa ($Na^+$) and/or PBFI-AM ($K^+$) dyes were added. Ion content in was analyzed after 4h by flow cytometry using fluorescence histogram plots **(A)** or contour plots to determine the relationship between alteration in intracellular $K^+$ and $Na^+$ and **(B)**. In **C**, alterations in plasma membrane potential were determined using DiBAC4(3) incubated 30 minutes prior to flow cytometry analysis. Alterations in plasma membrane potential are presented as changes in mean fluorescence intensity. In **D**, caspase 3/7-like activity was performed as explained in Figure 1C.

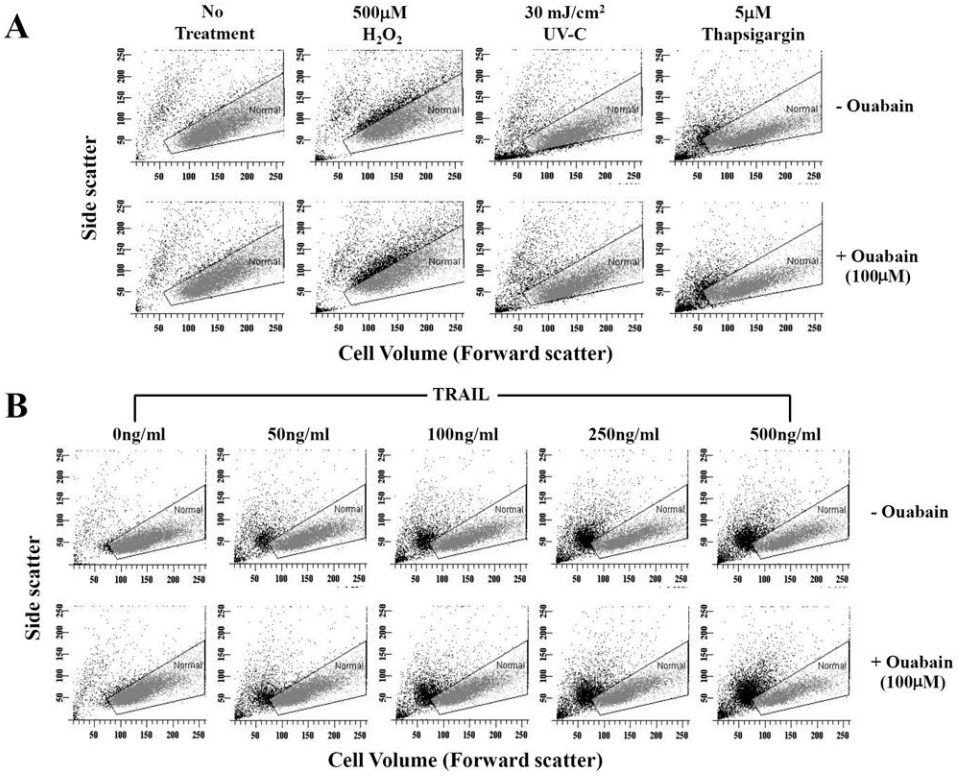


**Fig. 3. Effect of ouabain on apoptosis induced by various stimuli**

Apoptosis was induced by addition of $H_2O_2$, thapsigargin, UV-C **(A)** or with increasing concentrations of TRAIL **(B)** for 4h in the presence or absence of ouabain. Cells were analyzed for changes in their cell size as explained in Figure 1A. Shown are single representative experiments from three independent experiments.

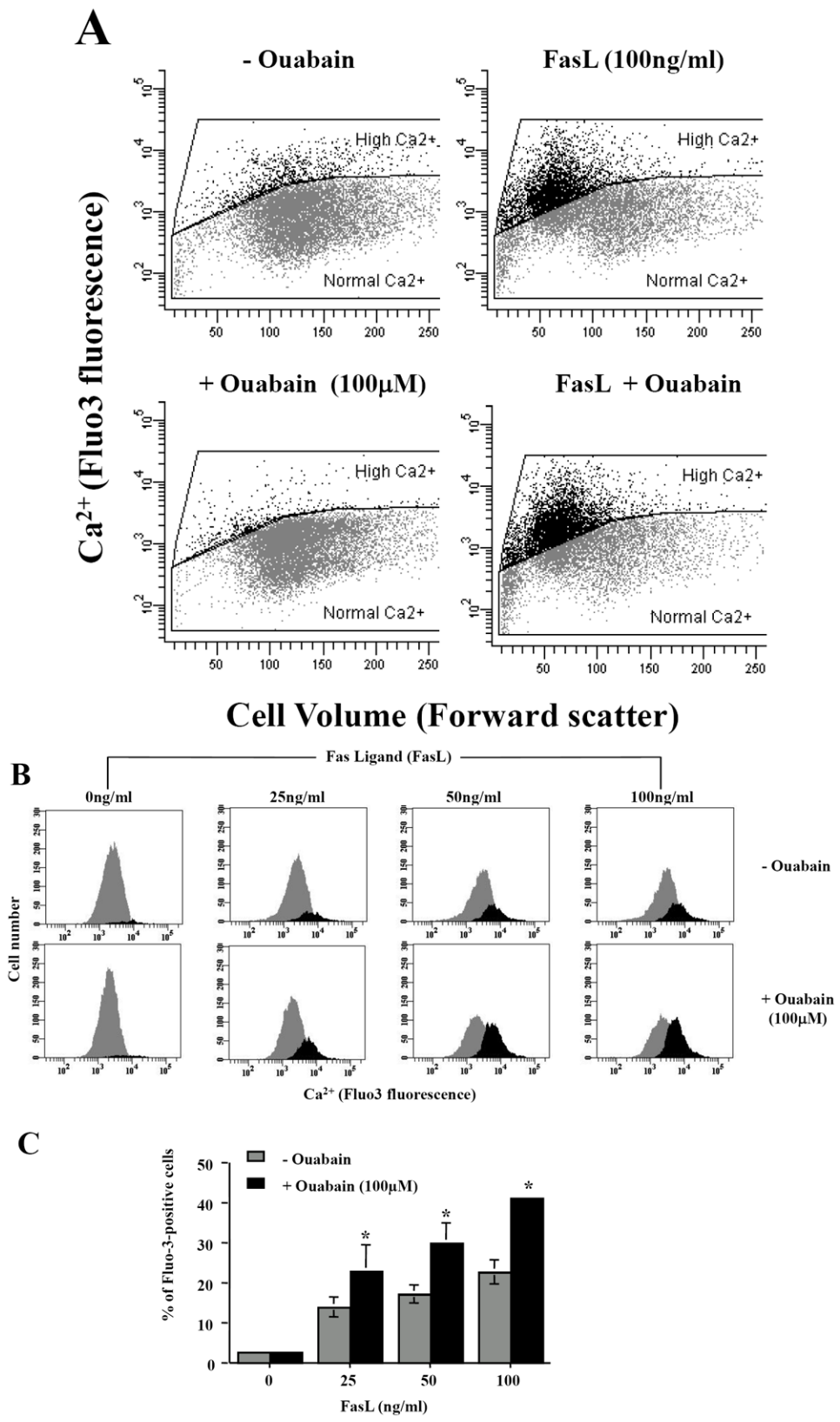


**Fig. 4. Effect of ouabain on cell $Ca^{2+}$ homeostasis in FasL-induced apoptosis**

Apoptosis was induced in Jurkat cells by addition FasL for 4h. Cells were loaded with Fluo-3 AM 30min prior to flow cytometry analysis. Intracellular $Ca^{2+}$ content was analyzed in either Fluo-3 fluorescence versus forward scatter dot plots **(A)** or Fluo-3 fluorescence histograms **(B)**. Shown are either a single representative experiment from three independent experiments, **(A** and **B)**, or averaged values ± SEM from three independent experiments **(C)**. Asterisks (*) indicate statistical significance ($P<0.05$) between the two experimental conditions at each concentration of FasL.

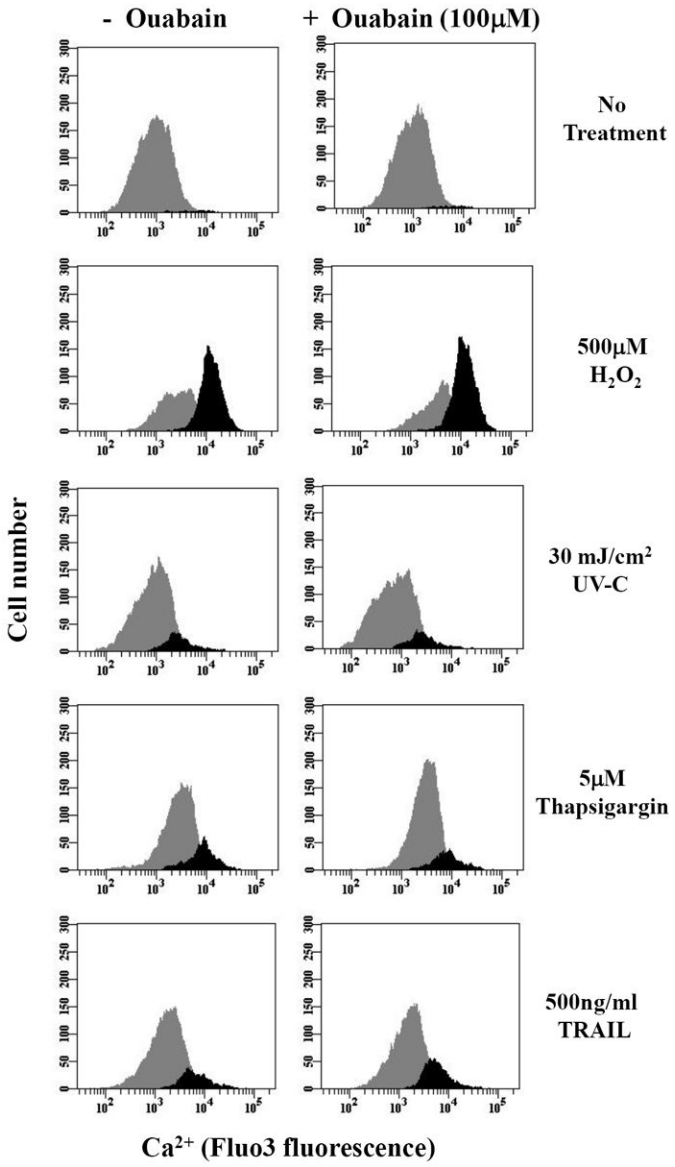


**Fig. 5. Effect of ouabain on alterations in intracellular $Ca^{2+}$ homeostasis induced by various apoptotic stimuli**

Apoptosis was induced in by addition of $H_2O_2$, TRAIL, thapsigargin or UV-C for 4h in the presence or absence of ouabain. Intracellular $Ca^{2+}$ content was analyzed as explained in Figure 4B. Shown are single representative experiments from three independent experiments.

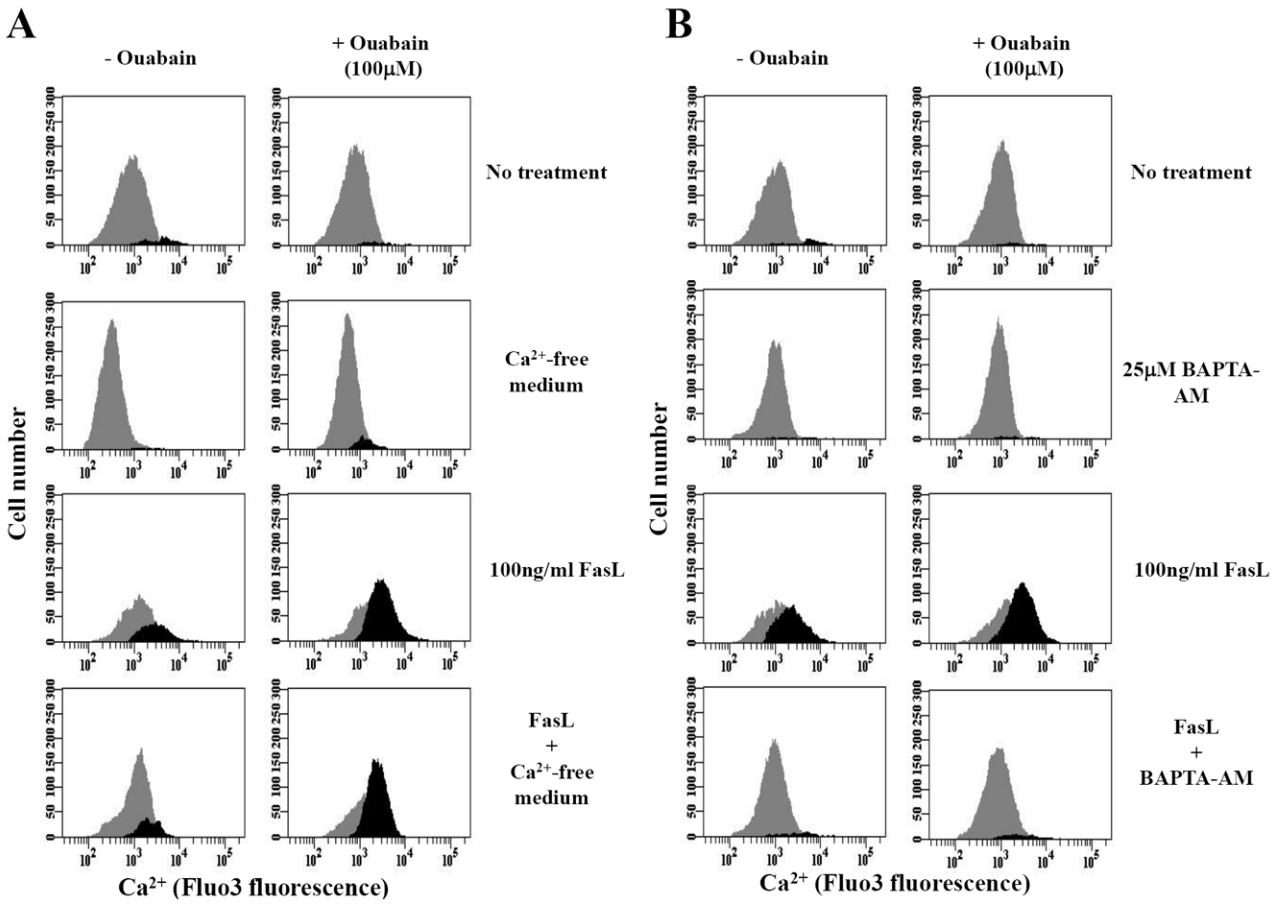


**Fig. 6. Effect of $Ca^{2+}$-free medium and BAPTA-AM on FasL-induced increase in intracellular $Ca^{2+}$ homeostasis in the presence or absence of ouabain**

Apoptosis was induced by addition of 100ng/ml FasL for 4h in the presence or absence of ouabain ouabain under experimental conditions involving $Ca^{2+}$-free medium **(A)**. Alternatively, cells were treated with BAPTA-AM for 30 min prior to addition of FasL **(B)**. Intracellular Ca2+ content was analyzed as explained in Figure 4B. Shown are single representative experiments from three independent experiments.

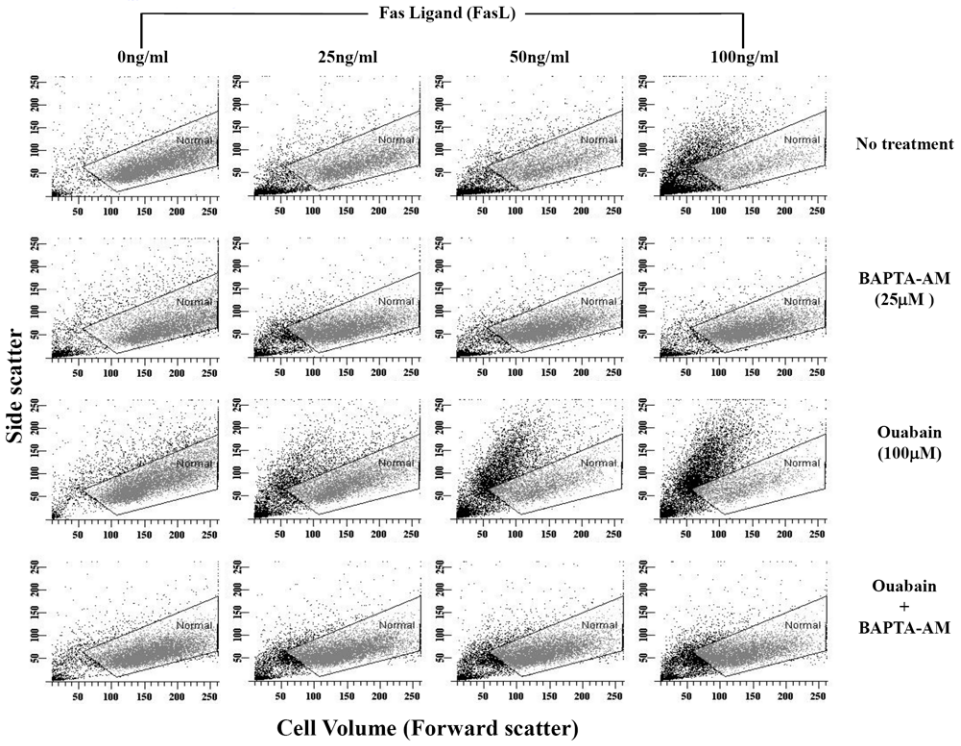


**Fig. 7. Effect of BAPTA-AM on FasL-induced apoptosis in the presence or absence of ouabain**

Apoptosis was induced in Jurkat cells by addition of FasL for 4h in the presence or absence of ouabain at a final concentration of 100μM. Thirty minutes prior to addition of FasL, cells were treated with BAPTA-AM. Cells were analyzed for changes in their cell size as explained in Figure 1A. Shown are single representative experiments from four independent experiments.

## TABLE 1

Effect of ouabain on FasL-induced apoptosis.

| | % "apoptotic" cells | | | |
|---|---|---|---|---|
| | **0ng/ml FasL** | **25ng/ml FasL** | **50ng/ml FasL** | **100ng/ml FasL** |
| - Ouabain | 15.7 ± 1.2 | 31.1 ± 2.2 | 36.0 ± 3.4 | 41.6 ± 2.3 |
| +100μM ouabain | 22.4 ± 1.6 | 41.2 ± 1.7* | 49.3 ± 1.2* | 61.9 ± 1.2* |

Results represent the means ± the standard error of five independent experiments. % of apoptotic cells reflects the percentage of shrunken cells determined as shown in Figure 1A. Asterisks indicate statistical significance (at $P<0.05$) between mean values of control cells (- Ouabain) and ouabain treated cells.

**TABLE 2**

Effect of ouabain on TRAIL-induced apoptosis.

| | % "apoptotic" cells | | | | |
|---|---|---|---|---|---|
| | **0ng/ml TRAIL** | **50ng/ml TRAIL** | **100ng/ml TRAIL** | **250ng/ml TRAIL** | **500ng/ml TRAIL** |
| - Ouabain | 16.5 ± 1.5 | 29.1 ± 1.3 | 43.4 ± 1.1 | 48.0 ± 1.9 | 61.5 ± 1.5 |
| + 100μM Ouabain | 22.2 ± 1.6 | 39.9 ± 1.7 | 56.3 ± 1.6* | 59.0 ± 1.9* | 70.5 ± 1.5* |

Results represent the means ± the standard error of three independent experiments. % of apoptotic cells reflects the percentage of shrunken cells determined as shown in Figure 3B. Asterisks indicate statistical significance (at $P<0.05$) between mean values of control cells (- Ouabain) and ouabain treated cells.

**TABLE 3**

Effect of ouabain on apoptosis-induced $Ca^{2+}$ increase.

| | % of cells with High intracellular $Ca^{2+}$ | | | | |
|---|---|---|---|---|---|
| | **No treatment** | **500μM $H_2O_2$** | **5μM Thapsigargin** | **30mJ/cm$^2$ UV-C** | **500ng/ml TRAIL** |
| - Ouabain | 1.40 +/- 0.23 | 57.0 +/- 6.19 | 2.37 +/- 0.43 | 1.63 +/- 0.37 | 26.0 +/- 6.90 |
| + 100μM Ouabain | 0.97 +/- 0.07 | 58.4 +/- 6.21 | 6.47 +/- 0.78 | 0.70 +/- 0.21 | 31.2 +/- 6.83 |

Results represent the means ± the standard error of three independent experiments. % cells with High intracellular $Ca^{2+}$ was determined as shown in Figure 4. None of these values are significant ($P<0.05$) when the individual treatments +/- ouabain are compared.

## TABLE 4

Effect of BAPTA-AM on the potentiation of FasL-induced apoptosis by ouabain.

| | % "apoptotic" cells | | | |
|---|---|---|---|---|
| | **0ng/ml FasL** | **25ng/ml FasL** | **50ng/ml FasL** | **100ng/ml FasL** |
| No treatment | 24.5 ± 1.6 | 48.5 ± 0.8 | 54.2 ± 2.8 | 57.7 ± 4.4 |
| BAPTA-AM | 32.5 ± 1.9 | 37.8 ± 3.0$^{\#}$ | 38.7 ± 3.7$^{\#}$ | 40.5 ± 3.7$^{\#}$ |
| Ouabain | 28.5 ± 2.1 | 52.1 ± 3.0 | 63.6 ± 1.8 | 68.5 ± 2.2 |
| BAPTA-AM + Ouabain | 32.8 ± 1.2 | 37.7 ± 1.0$^{*}$ | 42.8 ± 2.2$^{*}$ | 46.5 ± 4.4$^{*}$ |

Results represent the means ± the standard error of four independent experiments. % of apoptotic cells reflects the percentage of shrunken cells determined as shown in Figure 7. * and # indicate statistical significance (at $P<0.05$) between mean values of cells treated with FasL (*) ± Ouabain (#) and cells preloaded with the intracellular $Ca^{2+}$ scavenger BAPTA-AM.