Stimulation of Kv1.3 Potassium Channels by Death Receptors During Apoptosis in Jurkat T Lymphocytes

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Running title: Fas induced potassium currents
Summary

The loss of intracellular potassium is a pivotal step in the induction of apoptosis but the mechanisms underlying this response are poorly understood. Here we report caspase-depdendant stimulation of potassium channels by the Fas receptor in a human Jurkat T cell line. Receptor activation with Fas ligand for 30 minutes increased the amplitude of voltage-activated potassium currents two fold on average. This produces a sustained outward current, ~10 pA, at physiological membrane potentials during Fas ligand-induced apoptosis. Both basal and Fas ligand-induced currents were blocked completely by toxins that selectively inhibit Kv1.3 potassium channels. Kv1.3 stimulation required the expression of Fas-associated death domain (FADD) protein and activation of caspase 8, but did not require activation of caspase 3 or protein synthesis. Furthermore, Kv1.3 stimulation by Fas ligand was prevented by chronic stimulation of protein kinase C with 20 nM phorbol 12-myristate 13-acetate (PMA) during Fas ligand treatment, which also blocks apoptosis. Thus, Fas ligand increases Kv1.3 channel activity through the same canonical apoptotic signaling cascade that is required for potassium efflux, cell shrinkage and apoptosis.
Introduction

Programmed cell death, or apoptosis, is an essential process for normal tissue homeostasis and immune system regulation. One of the early features of apoptosis is the loss of potassium ions and the accompanying cell shrinkage, also termed apoptotic volume decrease (AVD) (1,2). Blocking efflux of potassium from cells triggered to undergo apoptosis by culturing them in high potassium media is sufficient to prevent apoptosis (3-7). Although changes in the ionic environment appear to be required for the activation of the apoptotic machinery, such as caspases, nucleases and cytochrome c release (4,8), neither the signaling pathway that triggers potassium efflux after the initiation of apoptosis nor the channels that allow the potassium ions to pass out of the cell have been identified yet. We were interested in the signaling pathway that results in potassium efflux during apoptosis. We used Fas ligand-mediated apoptosis of the well-characterized human lymphoma Jurkat-T cell line to study the mechanism of potassium efflux and the signaling molecules involved in this important feature of the death pathway.

Apoptosis is the central regulatory feature of the immune system, controlling lymphocyte maturation, receptor repertoire selection, and homeostasis (9). The elimination of most T cells after the peak proliferative, clonal expansion phase of an immune response is orchestrated by death receptor-associated apoptosis. A subset of the receptor for tumor necrosis factor (TNF-R) family members are the so called “death receptors” that transduce death signals. In particular, the death receptor Fas (CD95) is a 45 kDa protein that induces T cell death upon binding to its natural ligand Fas (10). Death receptors harbor in their structure death domains (DD) that are necessary to signal
apoptosis (11). Within seconds of Fas ligand binding to the Fas/CD95 receptor, trimerization and receptor activation occurs. This is rapidly followed by the formation of a death-inducing signaling complex (DISC) (12), which recruits the scaffolding protein, Fas associated death domain protein (FADD), to the death domain of the activated Fas receptor. FADD in turn initiates a caspase cascade that begins with the mobilization and activation of pro-caspase 8 (13). Active caspase 8, in turn, cleaves and activates pro-caspase 3. The active effector caspase 3 is a semi-selective protease, which disrupts normal cell function by cleaving key structural proteins and signaling molecules that ultimately results in cellular destruction. T lymphocytes are known to express several classes of potassium channels (14), that are known to be involved in cell fate decisions (15,16). Here we report a Fas-induced increase in the voltage-dependent potassium current carried by Kv1.3 channels. We also show that this increase in potassium channel activity, like several other components of apoptosis, is dependent on Fas receptor activation, recruitment of FADD and caspase 8 activation.
Experimental Procedures

Reagents and Protocol of Cell Culture and Treatments

Jurkat T cells, E6.1 (human lymphoma) were cultured at 37°C and 7% CO₂ in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 4 mM glutamine, 75 units/ml streptomycin and 100 units/ml penicillin. FADD deficient Jurkat cells were kindly provided by Dr. J. Blenis (Harvard) and incubated under the same conditions described for Jurkat T cells. Cells (~ 1.5 X 10⁶/ml cells) were incubated in the presence or absence of 2 µg/ml Fas ligand or 2 µg/ml Fas antibody (Kamiya Biomedical Company, Seattle, WA). Caspase inhibitors were incubated with cells at 100 µM of either caspase 3 inhibitor, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (DEVD), caspase 8 inhibitor, benzyloxycarbonyl-Ile-Glu-Thr-Asp- fluoromethyl ketone (IETD) or broad spectrum caspase inhibitor, (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone) (Z-VAD) (Kamiya Biomedical Company, Seattle). Protein kinase C activator, phorbol ester 12-O-tetradecanoylphorbol 13-acetate (20 nM) (PMA) (Calbiochem, La Jolla, CA) was incubated with cells for 30 minutes. Protein kinase C inhibitor Gö6976 (Calbiochem, La Jolla, CA) was used at a concentration of 2.5 µM. Both PMA and Gö6976 were freshly dissolved in DMSO (final DMSO concentration never exceeded 0.1% in the culture medium).

Electrophysiology

Jurkat cells were bathed in a salt solution at 20-24°C and voltage-clamped to -80 mV in the whole-cell recording mode with an Axopatch 200 amplifier (Axon Instruments, Foster City, California). The bath solution for these recordings contained (in mM) 5 KCl,
140 tetramethyl-ammonium chloride, 1 CaCl₂, 1 MgCl₂, 10 HEPES and 5 glucose at pH 7.3. The pipette solution contained (mM) 140 potassium gluconate, 2 MgCl₂, 2 EGTA, 10 HEPES and 5 glucose at pH 7.4. Immediately prior to recording Jurkat cells were collected by gentle centrifugation and resuspended in bath solution. Potassium currents were evoked by a range of 100 ms voltage steps from –60 mV to 80 mV from a holding potential of –80 mV. The sustained currents were evoked by 9.8 s step depolarizations between –50 and +30 mV. The current was measured after 8 seconds recording.

Channel block was achieved by bath perfusion (2 ml/min) of 5 nM (L-a,b-Diaminopropionyl₂²)-Stichodactyla helianthus Neurotoxin (Shk Dap₂²) (Bachem, King of Prussia, PA) or 50 nM Margatoxin (Alomone labs, Jerusalem, Israel).

**Fluorescence Activated Cell Sorting (FACS)**

Changes in cell volume were assessed by flow cytometry using a Becton Dickinson FACSort. Five thousand cells per ml were used per sample. Excitation with a 488 nM argon laser was used to determine light scatter. Changes in cell volume for each population were determined with FACS by measuring forward light scatter. The forward scatter versus cell number values were analyzed with CellQuest software.

Acute changes in plasma membrane potential were measured by FACS using DiBAC₄(3) (Molecular Probes). Jurkat cells were incubated in RPMI 1640 containing 150 nM DiBAC₄(3) (Molecular Probes, Eugene OR) for 10 minutes at 37°C, 7% CO₂ atmosphere and immediately examined by flow cytometry using a Becton Dickinson FACSort using argon laser excitation at 488 nm and 530 nm of emission.

For analysis of DNA degradation cells were fixed in cold 70% ethanol overnight, pelleted, washed once in phosphate-buffered saline (PBS) and resuspended in PBS.
containing 20 µg/ml propidium iodide (Sigma). Cells were examined by flow cytometry for propidium iodide fluorescence and the percentage of degraded DNA was determined by the number of cell displaying sub-diploid DNA divided by the total number of cells examined.

Western Blot Analyses

Jurkat lysate preparation was done as previously described (17). Briefly, cells were lysed a buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, and protease inhibitors (Complete™). Cells were then disrupted with a 25-gauge needle, centrifuged at 16,000 x g for 15 min, and protein normalized among the different treatments. Samples were run onto 4 - 20% tris-glycine gels and exposed to antibodies after blotting. Monoclonal anti-caspase-8 was from Calbiochem (San Diego, CA); rabbit polyclonal anti-procaspase-3 and monoclonal anti-processed caspase-3 were from Cell Signaling and Zymed, respectively; monoclonal anti-FADD was from BD Biosciences-Pharmingen (CA). HRP-linked secondary anti-mouse IgG or anti-rabbit IgG were from Amersham (Piscataway, NJ).
Results

Fas ligand increases voltage-activated potassium current before cell shrinkage

The voltage-activated potassium conductance of Jurkat T lymphocytes was measured with the conventional whole-cell voltage-clamp technique and normalized to cell capacitance to control for cell size. Figure 1 shows representative currents elicited from control untreated cells (B) and Jurkat T cells incubated with 2 µg/ml Fas ligand for 30 minutes (C). The potassium current density of Fas ligand treated cells was approximately two fold larger on average compared to the untreated controls at all test potentials that elicited a current. Because apoptosis occurs stochastically in response to all death signals cells were incubated with a high concentration of Fas ligand (2 µg/ml) to accelerate the initiation and progression of apoptosis. Cells treated with Fas ligand for less than twenty minutes had currents of the same average amplitude as untreated cells (not shown). Cell shrinkage is a hallmark of apoptosis that is associated with potassium efflux from the cell (18). The time course of cell shrinkage was analyzed by flow cytometry and compared to the onset of potassium efflux in different cell populations. Although treatment of Jurkat T lymphocytes for 30 minutes with Fas ligand (2 µg/ml) was not sufficient for significant cell shrinkage compared to untreated populations, 58% of the cell population exhibited cell shrinkage after 1 hour of incubation (data not shown). Thus, the delayed increase in potassium current produced by Fas ligand occurred early enough to account for the subsequent reduction in cell volume.
Kv1.3 channels conduct the Fas-induced potassium current

The voltage-activated potassium current observed after Fas ligand stimulation had similar kinetics to channels of the Shaker family of delayed-rectifier channel proteins (19), so we tested whether the current was conducted through Kv1.3 channels, which activated lymphocytes are known to express (20,21). At nanomolar concentrations, the scorpion toxin, margatoxin and the mono-substituted analogue of a sea anemone toxin, (L-a, b-Diaminopropionyl\textsuperscript{22})-Stichodactyla helianthus Neurotoxin (ShK Dap\textsuperscript{22}) are selective blockers of Kv1.3 channels (22,23). Potassium currents from Jurkat cells treated with Fas ligand for 30 minutes were recorded before and after bath perfusion with margatoxin or ShK Dap\textsuperscript{22}. A representative family of Fas-induced current traces is shown in Figure 2 before (A) and after bath application of ShK Dap\textsuperscript{22} (5 nM) (B). Figure 2C shows the current-voltage relationship derived from untreated cells, cells treated with Fas ligand for 30 minutes and Fas ligand treated cells after bath perfusion with margatoxin (50 nM).

Margatoxin (50 nM) blocked the majority of the Fas induced current (89% ± 2.8, n = 6). The Kv1.3 selective sea anemone toxin analogue, ShK Dap\textsuperscript{22} (5 nM) also inhibited the majority (83% ± 6.9, n = 4) of the Fas-induced potassium current. Thus, our data strongly suggest that Kv1.3 is the predominant voltage-activated channel activated by Fas ligand in Jurkat cells. Therefore we examined the mechanism of Kv1.3 stimulation by Fas ligand.

Fas increases Kv1.3 channels through the Fas receptor

Jurkat T cells incubated with either Fas ligand or Fas receptor antibody for 30 minutes showed a doubling of the voltage-activated potassium current amplitude (Fig. 3A). In
contrast, the non-stimulating Fas antibody (Zb-Ab, 5 µg/ml), which binds to the Fas receptor but does not result in receptor activation, did not increase potassium current (Fig. 3A). Thus, the increase in current required Fas receptor activation. Furthermore, decreasing the dose of Fas ligand produced even longer delays before the increase in current amplitude could be detected. Only one out of four cells treated with 200 ng/ml Fas ligand for 30 minutes showed potassium currents over 20 pA/pF. After 45 minutes however, five out of six cells showed currents over 20 pA/pF (data not shown).

Kv1.3 channel stimulation by Fas signaling does not require de novo protein synthesis

De novo protein synthesis is not required for Fas ligand-induced apoptosis of Jurkat T lymphocytes (24), but the Fas ligand-induced increase in Kv1.3 potassium current required at least 20 minutes incubation with Fas ligand. Therefore, we investigated whether the delay reflected a requirement for de novo protein synthesis. Jurkat T lymphocytes were incubated with the protein synthesis inhibitor, cycloheximide (5 µM), for 5 minutes prior to addition of Fas ligand, and cycloheximide was present during the subsequent 30 minutes of Fas ligand treatment. Despite inhibition of protein synthesis, Fas ligand produced an increase in current comparable to control Jurkat T cells treated with Fas ligand for 30 minutes (Fig. 3B). Thus, like Fas ligand-induced apoptosis, the Fas ligand-induced potassium current increase did not require de novo protein synthesis. The cytoplasmic signaling events downstream of Fas receptor activation and the subsequent caspase cascade that transduces apoptosis have been well characterized (10,25). To investigate the mechanism of Kv1.3 stimulation triggered by Fas receptor activation we used both genetic and pharmacological approaches.
Recruitment of FADD is necessary for the Fas-induced potassium increase

Fas receptor activation stimulates the recruitment of the scaffolding protein, Fas-associated death domain protein (FADD), to the intracellular domain of the activated Fas receptor, which is necessary for the initiation of the caspase cascade (12). To test whether FADD is necessary for signaling the increase of Kv1.3 channel activity we used FADD-deficient Jurkat T cells that express a low level of inactive FADD protein (26). Figure 4A confirms that in FADD-deficient T cells neither cleavage of procaspase 8 to the active caspase 8 protein nor cleavage of procaspase 3 to active caspase 3 protein occurs after Fas ligand stimulation. We treated the FADD deficient cells with Fas ligand for 30 minutes and measured the potassium currents as described above. The Fas ligand-induced potassium current increase was abolished in the FADD-deficient cells (Fig. 4B).

Caspase 8 is necessary for the Fas-induced potassium current increase

Activation of FADD by the Fas receptor leads to activation of caspase-8. To evaluate the role of caspase-8 in the potassium channel activation pathway we first used two pharmacological inhibitors: the broad spectrum caspase inhibitor, ZVAD (10 and 100 µM) and the selective peptide inhibitor of caspase-8, IETD (100 µM) (27,28).

Pharmacological inhibition of caspase 8 prevented the Fas-induced increase in potassium currents (Fig. 5A). Both ZVAD and IETD (100 µM) blocked the DNA degradation when Jurkat T cells were stimulated with Fas ligand (2 µg/ml) confirming that these inhibitors are effective at blocking caspase activity under these conditions (Figure 5D). We also studied a lymphocyte cell line deficient in caspase 8 to define the signaling pathway.
Figure 4A confirms that capase 8 deficient cells do not contain any detectable cleaved, active caspase 8 protein and the conversion of procaspase 3 to active caspase 3 protein does not occur. The absence of caspase-8 prevented the Fas ligand-induced increase in potassium current (Fig 4B). Thus, selective block of caspase-8 activity either pharmacologically or by genetic mutation of caspase-8 prevented the Fas-induced increase of potassium currents in Jurkat T cells.

Caspase-3 is not required for the potassium current increase

The executioner caspase, caspase-3, is activated directly by caspase-8. Caspase-3 then cleaves key structural and signaling molecules resulting in extensive protein breakdown and cellular destruction during apoptosis. We were interested to discover whether caspase-3 activity was necessary for the observed increase in potassium currents. To investigate this we used a selective caspase-3 peptide inhibitor (DEVD) (27). Despite incubation with the caspase-3 inhibitor DEVD (100 µM) for 30 minutes, Fas ligand produced the same increase in potassium current as it did on control cells (Fig. 5B). We tested whether DEVD was an effective caspase blocker when cells had been triggered to undergo apoptosis with the relatively high Fas ligand concentrations used in these experiments. We used flow cytometry to analyze Jurkat T cell DNA content and found that pre-treating cells with DEVD (100 µM) for 30 minutes was sufficient to block DNA degradation induced by Fas ligand (2 µg/ml). Figure 5 C shows a representative frequency histogram of propidium iodide fluorescence showing the DNA content of untreated control cells, Fas ligand treated cells and DEVD (100 µM) and Fas ligand (2
µg/ml) treated cells. Thus, caspase-8 but not caspase-3 is necessary for the Fas-induced increase in potassium current.

*Protein kinase C prevents Fas-induced potassium currents*

Previous studies have shown that activation of protein kinase C with phorbol esters can inhibit Fas-induced apoptosis at signaling sites upstream of caspase-8 activation, by acting at the Fas receptor to prevent the recruitment of signaling/scaffolding proteins required for death signaling (17). Figure 6A illustrates our finding that Jurkat T lymphocytes treated with the protein kinase C activator, PMA (20 nM), were protected from Fas-induced cell shrinkage, which is a reflection of potassium loss from the cells. We were interested in whether protein kinase C activation could also inhibit the Fas-induced increase in potassium current. Jurkat T lymphocytes that were incubated with both PMA (20 nM) and Fas ligand (2µg/ml) for 30 minutes did not show any Fas-induced potassium current increase (Fig. 6B). The PKC-dependent modulation of the Fas signaling pathway that is known to block cell shrinkage and apoptosis also blocked the increase in potassium currents. It is also worth noting that Fas-induced potassium current rapidly decreased in amplitude after bath application of PMA (20nM) (control, 19 ± 7% current decrease in 15 minutes, n = 5; PMA, 53 ± 4% current decrease in 15 minutes, n = 8). These data suggest that protein kinase C may have actions on the channel proteins in addition to its upstream targets in the Fas receptor signaling pathway (39). Conversely, it has been shown that inhibition of PKC enhances Fas-induced cell shrinkage and apoptosis. Figure 6A shows changes in cell size of Jurkat T cells treated with a PKC inhibitor, Gö6976 (2.5 µM) for 30 minutes, illustrating the stimulatory effect of Gö6976
on Fas-induced cell shrinkage. We investigated whether inhibition of protein kinase C could also affect the amplitude of potassium currents. To this end we inhibited PKC by incubation of Jurkat T lymphocytes with Gö6976 (2.5 µM) for 30 minutes. This inhibition of PKC resulted in a significant increase in the normalized potassium current amplitude (P < 0.017) (Fig. 6B).

*Fas ligand dependent changes in ionic properties of Jurkat T cells*

The results presented above demonstrate that Fas ligand increases the amplitude of Kv1.3 currents in response to imposed depolarizations under voltage-clamp, but Jurkat T cells do not fire action potentials, so how are the channels activated during apoptosis? It has been reported that Jurkat T cells also depolarize prior to shrinking (34). We have confirmed that Fas ligand (2µg/ml), which increased potassium currents, also depolarized these cells (Fig. 7A). However, sustained depolarization not only activates Kv1.3 channels, it also inactivates them. Consequently we investigated whether Fas ligand stimulation increased the steady-state current. Figure 7B shows a family of traces evoked from a series of depolarizations from −50 to 30 mV for 9.8 seconds. The currents were measured after prolonged activation of 8 seconds (after the current has fully inactivated, τ_{inactivation} ~ 1 second). The mean currents, measured at 8 seconds, were plotted against membrane potential (see inset). The mean steady-state current of Fas ligand treated Jurkat T cells was significantly larger than those measured in control, untreated cells at all voltages tested (P < 0.0003). This sustained current remained sensitive to Shk Dap^{22} (5 nM); 67% ± 4, n = 4, current measured at 8 seconds was blocked by bath perfusion of toxin.
Discussion

The efflux of potassium ions is a pivotal apoptotic event in a variety of cell types that occurs in response to diverse stimuli: for example, cortical neurons in response to ceramide (5), myeloblastic leukemic cells in response to ultra violet irradiation (29) and Jurkat T lymphocytes in response to Fas ligand (4,30). Indeed potassium efflux is so critical for apoptosis that when it is blocked by bathing cells in high extracellular potassium, apoptosis is also blocked (4,31). The goal of this study was to identify the signaling events that increase potassium efflux in Jurkat T cells triggered to undergo the apoptotic program by the cytokine, Fas ligand.

We show here that Fas receptor activation increases the amplitude of the voltage-activated potassium currents in a human Jurkat T cell line. This effect took over 20 minutes to appear, but did not require protein synthesis and preceded cell shrinkage. Under our conditions, all the voltage-activated current was carried by a single class, Kv1.3, of Shaker-related, delayed rectifier channel proteins. The Kv1.3 channel has been identified as the predominant voltage-gated potassium channel expressed in lymphocytes (21). T cells also express several other potassium channels (16,32), which might also be stimulated by Fas ligand, but their contribution to Fas ligand induced apoptosis in Jurkat T cells was not tested here. Other studies show that lymphocytes genetically deficient in Kv1.3 channels are protected from undergoing the apoptotic program in response to cytostatic drugs (33). Based on the idea that Kv1.3 channels contribute to potassium efflux during apoptotic volume decrease we tested whether Fas ligand-induced stimulation of Kv1.3 channels were sufficient for potassium efflux during apoptosis. We
incubated cells with a blocker of Kv1.3 channels and asked if it was sufficient to prevent cell shrinkage. When cells were incubated with Shk Dap22 (10 nM) and treated with Fas ligand (2 µg/ml) for 30 minutes the cells still shrank showing no significant protective effect from the presence of the toxin channel blocker (data not shown). We conclude from this that other channels may also be involved in potassium efflux, for example, the two-pore domain potassium channels that have recently been shown to play a role in apoptotic volume decrease (34). Here we have focused on the signaling mechanism for the Fas receptor induced increase in Kv1.3 activity.

The increase of the Kv1.3 current amplitude in response to Fas ligand required the same signaling cascade as other cellular hallmarks of apoptosis, such as caspase activation (35), and cell shrinkage (17). Thus, either loss of FADD and the death receptor complex or loss of the downstream effector, caspase 8, completely prevented the increase in Kv1.3 activity. Furthermore, activation of protein kinase C (PKC) activity, which attenuates Fas-induced apoptosis at the Fas receptor level (36,37), prevented the increase in Kv1.3 channel activity. PMA also enhanced the rundown of both the Fas ligand-activated and control currents, suggesting that PKC could also act directly on potassium channels. Conversely, inhibition of PKC with Gö6976, which exacerbates the progression of apoptosis (17), increased the Kv1.3 current amplitude of Jurkat T cells even in the absence of Fas ligand, which also supports a more direct action of PKC on the channels. In contrast, inhibition of the executioner caspase, caspase 3, even with fairly non-specific peptide inhibitors, did not prevent the Fas-induced increase in Kv1.3 activity. This agrees with a recent report that caspase-3 activation is downstream of potassium efflux (4). Thus, Kv1.3 stimulation is a relatively early event in the apoptotic program and is
consistent with the postulated role of potassium efflux in contributing to the reduction in cytoplasmic ionic strength, which contributes to the activation of many of the executioner enzymes that carry out degradation. The only conundrum in this scenario is how a voltage-activated channel contributes to potassium efflux in a cell that does not fire action potentials.

We have used the voltage-clamp technique to measure the maximal available current in response to artificially imposed test depolarizations. Fas receptor activation increased the peak currents measured at 100 msec and also sustained currents measured at 8 seconds by approximately two fold at all voltages that elicited a current. In lymphocytes the net amount of channel activity will be determined by the cells’ resting membrane potential. Resting membrane potentials more positive than -40 mV will activate the channels, but as the membrane potential becomes more depolarized (positive) more channels will become unavailable due to steady-state inactivation of the channels. In many cells these two properties of activation and inactivation overlap so there is a window of membrane potential where the channels are activated but not completely inactivated, and this produces a steady potassium efflux. Thus, potassium efflux would require three coincident steps before it would become significant: Kv1.3 stimulation, inhibition of the Na/K ATPase and membrane depolarization. Indeed early in apoptosis the Na-K ATPase is inhibited and this is coupled to membrane depolarization (35,38). This could explain the long initial delay but rapid progression of apoptosis once all these conditions are met. In the Jurkat cells the amplitude of the sustained current produced by Fas receptor activation is more than enough to produce rapid loss of cytoplasmic potassium ions. Using estimates taken from our averaged data, Fas ligand-stimulated cells have a steady-
state current of ~10 pA at a membrane potential of –20 mV, which corresponds approximately to an efflux of 6 × 10⁷ potassium ions per second. Calculating that a 10 µM diameter cell contains approximately 0.5 pL of cytoplasm and 4 × 10¹⁰ potassium ions, it would take only 10 minutes for all of the potassium ions to leave the cell in the absence of pumping. If each potassium channel passes 1 pA, then only 10 open channels would be required. Thus, even a voltage-activated potassium channel, such as Kv1.3, could account for the rapid and complete loss of potassium ions during apoptosis particularly if the cells were first depolarized and the Na/K ATPase was inhibited as has been recently reported (34). Although the sustained current is sufficient to account for cell shrinkage on the timescale examined here, it does not fully account for the Fas ligand-induced increased current amplitudes observed under voltage-clamp. There must be an additional increase in channel density or a reorganization of existing channels in the membrane to account for the increased currents. Alternatively the increased potassium current amplitude might result from an increased open probability due to channel modification such as a phosphorylation or dephosphorylation events. Caspases are selective proteases that cleave many signaling molecules that can result in the dysregulation of their activity. For example, caspase cleavage of protein phosphatase 2A switches it into a constitutively active mode (39). Because protein phosphatase 2A activity is known to increase Kv1.3 open probability it would be interesting to test its role in signaling the Fas-induced increase in potassium efflux (40). The signaling pathways downstream of caspase 8 that contribute to potassium channel activation remain unknown. A recent report shows that cytochrome C activates potassium channels when dialyzed into smooth muscle cells through the patch pipette (41). This presumably
mimics cytochrome C release from the mitochondria that is a critical step in initiating apoptosis that occurs down stream of caspase 8 activation (42-44).

In conclusion we show for the first time the signaling steps between the apoptotic stimuli and potassium efflux. The data presented here illustrates that the same signaling events downstream of Fas receptor activation that trigger the initiation of the caspase cascade also trigger potassium efflux by stimulating Kv1.3 channel activity.
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Figure Legends

Figure 1
The effect of Fas ligand on the potassium currents of Jurkat T cells. A: The voltage protocol used to elicit potassium currents in Jurkat T cells. Currents were evoked from a range of test potentials from -60 mV to 80 mV for 100 mseconds, from a holding potential of –80 mV. B: Representative currents from a control, untreated Jurkat T cell. C: Representative currents from a Jurkat T cell that had been treated with Fas ligand (2µg/ml) for 30 minutes.

Figure 2
The sensitivity of Fas ligand-induced potassium currents to invertebrate toxins. A: A representative family of potassium currents from a Jurkat T cell after incubation with Fas ligand for 30 minutes. The currents were elicited by a range of test potentials from -40 mV to 50 mV for 100 mseconds, from a holding potential of –80 mV. B: A family of potassium currents from the same cell as A after bath application of Shk-Dap22 (5 nM) using the same voltage protocol as A. C: A family of potassium currents from control, untreated cells (■), cells treated with Fas ligand (2 µg/ml) for 30 minutes (●) and Fas ligand treated cells after bath application of margatoxin (5 nM) (▲) were evoked by a range of test potentials between –40 mV and 50 mV from a holding potential of –80 mV. The current measured after 50 ms of test pulse, and normalized for cell capacitance, was plotted against the test potential.
Figure 3
The role of Fas receptor activation and inhibition of protein synthesis on the Fas ligand-induced increase in potassium conductance.  A:  The current elicited by a 40 mV test pulse was measured after 50 ms recording and normalized for cell capacitance for Jurkat cells treated for 30 minutes with Fas ligand, stimulatory Fas receptor antibody (Fas-Ab) or non-stimulatory Fas receptor antibody (Zb-Ab) and untreated control cells.  Both Fas ligand and Fas-Ab (2 µg/ml) treated cells were significantly larger than untreated or Zb-Ab (5 µg/ml) treated cells (P < 0.05).  B.  The bar graph shows the normalized current evoked by a test pulse to 40 mV.  Populations of Jurkat T cells had been treated with the Fas ligand for 30 minutes in the presence or absence of the protein synthesis inhibitor cycloheximide (5 µM).  Fas ligand treated cells in the presence of cycloheximide did not have significantly different current amplitudes from Fas ligand treated cells (P > 0.1).

Figure 4
The role of FADD and caspase 8 in the Fas ligand-induced increase in potassium currents of Jurkat T cells.  A:  Western blot analyses of caspase-8 and caspase-3 proteins from Jurkat cell, FADD-deficient cell, caspase 8-deficient parental cell, and caspase-8-deficient cell lysates.  The lysates were prepared as described in methods section after treatment of cells with or without Fas ligand for 1 hour.  Samples were run on a 4-20% Tris-glycine gels.  P52/54 and p32 bands for caspase-8 and caspase-3 indicate the pro-forms of the enzymes, respectively, and p41/43 and p12/17 the cleaved fragments for caspase-8 and caspase-3, respectively.  The panels on the left display non-treated cells and the right panels show Fas ligand-treated cells.  Blots are representative of at least four
independent experiments. B: FADD-deficient, caspase-8-deficient cells and vector control cells were treated with Fas ligand (2 µg/ml) for 30 minutes. The bar graph shows the normalized current evoked by a test pulse to 40 mV from untreated FADD-deficient, caspase-8-deficient and vector control cells and from Fas ligand treated FADD-deficient, caspase-8-deficient and vector control cells. The normalized current amplitudes of Fas ligand treated FADD- and caspase-8 deficient cells were significantly smaller than those of Fas ligand treated vector control cells (P < 0.005).

Figure 5
The effect of caspase inhibitors on the normalized potassium current amplitude of Fas ligand treated Jurkat cells. A: Jurkat T cells were treated with Fas ligand for 30 minutes after pre-incubation with either the broad spectrum caspase inhibitor (ZVAD, 100 µM) or the caspase-8 inhibitor (IETD, 100 µM) to block caspase activity. Potassium currents were evoked by a test pulse to 40 mV and then normalized for cell capacitance. Cells pre-treated with either ZVAD or IETD and stimulated with Fas ligand showed no significant difference in normalized current amplitude compared to un-stimulated controls (P ≥ 0.01). B: Jurkat T cells were pre-incubated with the selective caspase 3 inhibitor (DEVD, 100 µM) and then treated with Fas ligand for 30 minutes for comparison with cells that had not been exposed to a caspase inhibitor. The bar graph shows the normalized current amplitudes of control Fas ligand treated and caspase 3 inhibited, Fas ligand treated cells. Cells pretreated with DEVD and stimulated with Fas ligand showed a larger normalized current compared with un-stimulated controls (p = 0.03). Similarly, Fas ligand stimulated cells showed a significant increase in normalized
current compared to un-stimulated controls (P < 0.002). C. Jurkat T cells were either left untreated (control), or treated with Fas ligand (2 µg/ml) for 30 minutes, or pre-incubated with DEVD (100 µM) for 30 minutes and then treated with Fas ligand (2 µg/ml) for a further 30 minutes (Fas Ligand and DEVD). Cells were fixed and stained with propidium iodide for flow cytometric analysis of DNA content. The frequency histograms show the distribution of cells with normal DNA (to the right of the white line) and sub-diploid DNA content (left of the white line). D. Jurkat T cells were pre-incubated with caspase inhibitors ZVAD, IETD or DEVD (all at 100 µM) for 30 minutes, then treated with Fas ligand (2 µg/ml, FasL) for a further 90 minutes. The percentage sub-diploid DNA content (measured left of the white line) is shown in the bar graph for all the treatments.

Figure 6

The effect of protein kinase C on Fas ligand-induced cell shrinkage and potassium efflux in Jurkat T cells. A: Jurkat T-cells were pre-treated with or without a protein kinase C activator (PMA, 20 nM) or protein kinase C inhibitor (GÖ6976, 2.5 µM) for 30 minutes and with Fas ligand for 30 more minutes prior to analyses. The changes in the light scattering properties of the cell were determined by flow cytometry as described previously using a Becton Dickinson FACSort. A decrease in forward-scattered light correlates with cell shrinkage and is depicted by the blue line. B: The bar graph shows the normalized potassium current amplitudes of Jurkat T cells measured at 40 mV that have been pretreated with protein kinase C activators or inhibitors for 30 minutes and with Fas ligand for a further 30 minutes. The activation of protein kinase C (PMA, 20
nM) significantly blocked the increase in potassium currents in response to Fas ligand (2 µg/ml) (P < 0.005). Inhibition of PKC (Gö6976, 2.5 µM) resulted in a significant increase in potassium current (P < 0.02).

Figure 7
The effect of Fas ligand on the ionic properties of Jurkat T cells. A: The effect of Fas ligand on the plasma membrane potential of Jurkat T cells. Representative frequency histograms for control and Fas ligand treated cell populations are shown. The solid bar represents the median fluorescence of the control sample and cellular depolarization was observed by an increase in cellular fluorescence and is stated in each histogram compared to the value in the control sample. Cells were treated with Fas ligand (2 µg/ml) for 30 minutes and incubated with the plasma membrane potential indicator DiBAC4 30 minutes prior to time of examination. One ml of cells (10 000 cells per condition) was incubated with DiBAC4 (150 ng/ml) at 37°C, 7% CO2 atmosphere. Plasma membrane potential changes were measured by flow cytometry using a Becton Dickinson FACSort as described above and analyzed with CellQuest software. B: The effect of Fas ligand stimulation on the sustained potassium currents of Jurkat T cells. A family of currents evoked by 9.8 second step depolarizations from −50 mV to 30 mV. The current was measured at 8 seconds of recording and the mean current was plotted against voltage and is shown in the inset. The mean current ± s.e.m from Jurkat T cells treated with Fas ligand (2 µg/ml) for 30 minutes (●) and control, untreated cells (■) plotted against the test potential.
Figure 1

A

B

C
Figure 2

A

Fas ligand treated cell

100 pA
20 ms

B

Shk-Dap^{22} (5 nM)

100 pA
20 ms

C

pA/pF

- control n = 12
- Fas ligand n = 6
- Fas ligand + margatoxin n = 6
Figure 3

A

B

untreated control  Fas ligand  Anti Fas-Ab  Zb-Ab

n = 18  n = 10  n = 16  n = 5

pA/pF

0  20  40  60  80  100  120

cycloheximide  Fas ligand  Fas ligand + cycloheximide

n = 3  n = 5  n = 3

pA/pF
Figure 4

A

[Image showing Western blot analysis of FADD, p52/54 Pro-caspase-8, p41/43 Caspase-8, FADD, p32 Pro-caspase-3, p12/17 Caspase-3, Actin in Jurkat T-cells, Fadd-def, Parental C8, Caspase 8-def, Jurkat T-cells, Fadd-def, Parental C8, Caspase 8-def with and without FasL treatment.]

B

[Graph showing the results of the Western blot analysis with data points for vector, vector + Fas ligand, FADD, FADD + Fas ligand, caspase 8-, caspase 8- + Fas ligand, each with the number of replicates (n) indicated.]
Figure 5

A

![Graph A showing pA/pF values for different treatments with n values indicated for each group.]

B

![Graph B showing pA/pF values for different treatments with n values indicated for each group.]

Figure 5

C

Cell Number

Fas Ligand

Fas Ligand + DEVD

Propidium Iodide Fluorescence

D

% degraded DNA

control ZVAD IETD DEVD FasL FasL+ ZVAD FasL+ IETD FasL+ DEVD
Figure 6

A

![Graph showing cell number versus forward-scatter for different conditions: Control, Fas Ligand, PMA, PMA + Fas Ligand, Go6976, and Go6976 + Fas Ligand.](http://www.jbc.org/Downloaded from)

B

![Bar graph showing pA/pF values for different conditions: control, Fas ligand, Go6976, PMA, and PMA + Fas ligand.](http://www.jbc.org/Downloaded from)
Figure 7

A

![Graph showing cell number vs. fluorescence (DiBAC4) for control and Fas ligand conditions.]

- Control: 53%
- Fas Ligand: 87%

B

![Graph showing pA vs. potential (mV) with data points for Fas ligand (n = 8) and control (n = 6).]
Stimulation of Kv1.3 potassium channels by death receptors during apoptosis in Jurkat T lymphocytes
Nina M. Storey, Mireia Gomez-Angelats, Carl D. Bortner, David L. Armstrong and John A. Cidlowski

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