We address the specific role of cytoplasmic Ca\(^{2+}\) overload as a cell death trigger by expressing a receptor-operated specific Ca\(^{2+}\) channel, vanilloid receptor subtype 1 (VR1), in Jurkat cells. Ca\(^{2+}\) uptake through the VR1 channel, but not capacitative Ca\(^{2+}\) influx stimulated by the muscarinic type 1 receptor, induced sustained intracellular [Ca\(^{2+}\)]\(\text{rises, exposure of phosphatidylserine, and cell death. Ca}^{2+}\) influx was necessary and sufficient to induce mitochondrial damage, as assessed by opening of the permeability transition pore and collapse of the mitochondrial membrane potential. Ca\(^{2+}\)-induced cell death was inhibited by ruthenium red, protons, and cyclosporin A treatment, as well as by Bcl-2 expression, indicating that this process requires mitochondrial calcium uptake and permeability transition pore opening. Cell death occurred without caspase activation, oligonucleosomal/50-kilobase pair DNA cleavage, or release of cytochrome c or apoptosis inducer factor from mitochondria, but it required oxidative/nitritative stress. Thus, Ca\(^{2+}\) influx triggers a distinct program of mitochondrial dysfunction leading to paraptotic cell death, which does not fulfill the criteria for either apoptosis or necrosis.

Release of mitochondrial intermembrane proteins to the cytosol is a fundamental step in the cell death machinery during apoptosis and necrosis (1). Among other triggers, massive calcium uptake into isolated mitochondria induces the collapse of the mitochondrial membrane potential (\(\Delta \Psi_{m}\)) (1), the opening of the mitochondrial permeability transition pore or megachannel (PTP) and the release of proapoptotic factors such as cytochrome c (2) and/or apoptosis inducer factor (AIF) (3). It has been shown that high [Ca\(^{2+}\)]\(\text{rises is necessary for PTP opening in isolated mitochondria (1), but this [Ca}^{2+}\]\(] is not attained in the cytosol of intact cells. It has been proposed that mitochondrial Ca\(^{2+}\) overload in intact cells can trigger the opening of PTP and release of proapoptotic factors during neuronal ischemic and/or excitotoxic cell death (4, 5), but the downstream events that couple the rise in calcium to cell death are unknown. Mitochondrial Ca\(^{2+}\) overload would be a side result of the rise in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(c\)) (6), its extent critically depends on both the magnitude and duration of the [Ca\(^{2+}\)]\(c\) rise (7).

In the ischemic/excitotoxic stress model, the precise role of Ca\(^{2+}\) overload in neuronal cell death is too complex to trace (5). Stress stimuli often produce, in addition to Ca\(^{2+}\) entry through the plasma membrane, other effects, including (i) changes in mitochondrial membrane potential that can activate a variety of ion channels, (ii) metabolic changes that may lead to free radical production, (iii) activation of kinases, and (iv) release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores. Release of Ca\(^{2+}\) from the stores may be particularly relevant, as Ca\(^{2+}\) depletion of the endoplasmic reticulum (ER) blocks protein synthesis and is proapoptotic per se (8, 9).

Apoptosis is induced through Ca\(^{2+}\)-dependent pathways in several cell types. This raises the question of whether Ca\(^{2+}\) influx is sufficient for cell death. An apoptotic pathway involving up-regulation of the FasL gene has been extensively studied in T cells (10–12). This activation-induced cell death (AICD) pathway can be triggered by receptors that stimulate phosphoinositide turnover, such as the T cell receptor or the human muscarinic type 1 receptor (HM1R), which produce an increase in [Ca\(^{2+}\)]\(c\), by inositol 1,4,5-trisphosphate-mediated Ca\(^{2+}\) release from ER and capacitative Ca\(^{2+}\) entry. This is followed (>6 h) by calcium-dependent up-regulation of FasL mRNA expression (12, 13), FasL binding to Fas, and activation of buffered saline; PI, propidium iodide; PS, phosphatidylethanol; PTP, permeability transition pore; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species; TMRE, tetramethyl rhodamine methyl ester; TunEl, terminal deoxynucleotidyltransferase-mediated dUTP nick and labeling; VR1, vanilloid receptor subtype-1; WB, Western blot; Z-VAD, benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.
of caspases (14). Treatment with the sarcodopsinometric reticulum calcium ATPase blocker thapsigargin or with Ca\textsuperscript{2+} ionophores induces cell death in T cells (8); however, both treatments produce export of the ER (15, 16), which by itself can trigger apoptosis (9).

We address here the specific role of Ca\textsuperscript{2+} overload as a cell death trigger by transiently expressing vanilloid receptor type 1 (VR1) Jurkat J-HM1 cells. VR1–m was a recombinant protein, operated Ca\textsuperscript{2+} channel, which is naturally expressed in some neurons (17), peripheral blood T lymphocytes, and Jurkat cells (18, 19). Selective activation of VR1 with capsaicin allows for the control of Ca\textsuperscript{2+} influx through the plasma membrane and the study of specific effects of [Ca\textsuperscript{2+}], rises in our model. Since the T cell line used in this study also expresses HM1R, the effects of Ca\textsuperscript{2+} influx (through VR1) and Ca\textsuperscript{2+} release from the ER (through HM1R) can be directly compared. We find that sustained Ca\textsuperscript{2+} entry though VR1 but not Ca\textsuperscript{2+} release from ER induced by HM1R causes fast mitochondrial damage with loss of \Delta\psi\textsubscript{m} and caspase-independent, parapotic cell death. This cell death pattern is completely different from AICD, thus revealing that different programmed cell death pathways can coexist in the same cell and be induced by diverse stress stimuli.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**J-HM1–2.2 cells expressing the human HM1R have been previously described (20). Cells were maintained in Dulbecco’s modified Eagle’s medium (Bio-Whittaker Europe) supplemented with 5% heat-inactivated fetal calf serum, 1 mM-glutamine, 10 mM HEPES, penicillin-streptomycin (100 units/ml and 100 \mu g/ml; Bio-Whittaker Europe), and 0.5 mM-g G418 as a selection medium for expression of the HM1R.

**Antibodies and Reagents—**The anti-Fas (clone CH-11) monoclonal antibody was obtained from Medical and Biological Laboratories Co., Ltd., rabbit polyclonal anti-AIF was a kind gift from Dr. Susin (Institut Pasteur, Paris, France). Mouse monoclonal anti-cytochrome C was from Pharmingen (clone 6H2.B4). Human polyclonal anti-mitochondria Ab was from Dr. A. Serrano. Reagents used include 8-methyl-\textsuperscript{6}-dichlorodihydrofluorescein (DCFH\textsubscript{2}) oxidation by flow cytometry. The values of fluorescence at 530 mm; FL1) fluorescence in the cells excluding PI (605–545 mm; FL3) were used in experiments that required the absence of extracellular calcium. AIC15 monoclonal antibody anti-\beta-actin was from Sigma.

**Expression Vectors—**The pEG-FGF-VR1 plasmid was generated by subcloning the VR1 cDNA into the plasmid pEG-FGFPC1 (21) using the SacI and Kan restriction sites. For that purpose, the VR1 cDNA was subcloned into pCDNA3 (17) and was amplified by PCR with EcoTaq Plus polymerase (EcoGen, Ltd.), using the oligonucleotides VR1.1 (GG-GATTCGCTGACATGGAACAACGGGCTAGC) and VR1.2 (GCTCTAGACATGGAACAACGGGCTAGC) and VR1.2 (GCTCTAGACATGGAACAACGGGCTAGC) and VR1.2 (GCTCTAGACATGGAACAACGGGCTAGC). The construction of expression plasmids pEG355 and pEG human Bcl-2 (pEFhblc-2) was previously described (22). For some experiments involving measurement of 2',7'-dichlorodihydrofluorescein (DCF\textsubscript{2}) oxidation by flow cytometry in the FL1 channel, VR1 cDNA was subcloned into the pEGF-Myo-His B expression plasmid (pEG-VR1) (Invitrogen).

**Transfection Assays—**For transfection, cells in logarithmic growth were transfected with 20–30 \mu g of the pEG-FGF-VR1 plasmid by electroporation at 270 V/975 microfarads using the Gene Pulser II (Bio-Rad). Cells were analyzed 48 h after transfection. For the Western blot analysis, transfected cells were lysed for 10 min in ice-cold radioimmune precipitation lysis buffer supplemented with 1 mM phenylmethylsulfonfluoride. The cell lysate was centrifuged at 15,500 \times g for 5 min, and the supernatant protein was electrophoresed (25 \mu g/lane) on a 5% SDS-polyacrylamide gel and analyzed with an infrared Western blot (TBS, New England). For the flow cytometric analysis, cells were resuspended in PBS-propidium iodide (PI; 200 ng/ml) and then analyzed (Coulter Epics XL-MCL). Data analysis was performed with System II software (Coulter) by measuring the green (505–545 mm; FL1) fluorescence in the cells excluding PI (605–635 mm; FL3).

**Intracellular Calcium Measurements—**Single cell measurements of [Ca\textsuperscript{2+}] were performed by time-resolved ratiometric digital imaging fluorescence microscopy in cells loaded with the low affinity Ca\textsuperscript{2+} dye fura-4F. The dye was loaded into the cells by incubation with 4 \mu M fura-4F/AM (Molecular Probes, Inc., Eugene, OR) for 60 min at room temperature, in standard solution containing 145 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, 10 mM glucose, and 10 mM HEPES-sodium pH 7.4. Cells were then washed with fresh medium, resuspended at 10 \times 10\textsuperscript{5} cells/ml, and used for measurements.

**Coverslips (12-mm diameter) were coated with fibronectin by incubation (2 h, 37 °C) in PBS containing 20 \mu g/ml fibronectin (from human plasma; Sigma), followed by incubation in PBS containing 1% bovine serum albumin (1 h, 37 °C). Coverslips were mounted under the microscope (Olympus; Nikon) in 17°C chamber, and 10\textsuperscript{5} cells loaded with fura-4F were attached to the coverslip during 10 min. Test solutions were applied by continuous perfusion at 2–3 ml/min. This allowed >95% exchange of the medium bathing the cells within 5–10 s. For fluorescence measurements, cells were alternately epi-illuminated at 340 and 380 nm. Light emitted above 520 nm was recorded by an extended ISIS-M camera (Photonic Science, Borebridge, East Sussex, UK) and analyzed using an Applied Imaging Magic image processor (Sunderland, Tyne and Wear, UK) with 32-megabyte video RAM. Sixteen video frames of each wavelength were averaged by hardware with an overall time resolution of about 5 s for each pair of images at alternate wavelengths. Consecutive frames obtained at 340- and 380-nm excitation were ratiocinated pixel by pixel, and the Ca\textsuperscript{2+} was estimated using the following formula (23): [Ca\textsuperscript{2+}] = K\textsubscript{R}/(R – R\textsubscript{min}) (R\textsubscript{max} – R), where R is the ratio between the fluorescence emissions measured at 340- and 380-nm excitation, R\textsubscript{max} and R\textsubscript{min} are the ratios obtained at saturation of the dye with Ca\textsuperscript{2+} and in the absence of Ca\textsuperscript{2+}, respectively, K\textsubscript{R} is the dissociation constant for the dye (0.77 \mu M), and B is the ratio of the maximal (in the absence of Ca\textsuperscript{2+}) and the minimum (at saturation with Ca\textsuperscript{2+}) fluorescence emissions measured at 380-nm excitation. The values of R\textsubscript{max} and R\textsubscript{min} were determined in cells permeabilized to Ca\textsuperscript{2+} with ionomycin and perfused with media containing either no Ca\textsuperscript{2+} (5 mM EGTA) or 10 mM Ca\textsuperscript{2+}. These values were similar to the ones obtained with fura-2. These procedures have been described in detail (24). The cell density was described above for fura-4F loading except that bovine serum albumin (1 mg/ml) and EGTA (50 \mu M) were added to prevent spontaneous activation of cells during measurements. For the Ca\textsuperscript{2+}-free solution, CaCl\textsubscript{2} was omitted from the standard perfusion solution, and 100 \mu M EGTA was added.

**Assessment of Apoptotic Cell Death—**Detection and quantification of apoptosis at the single cell level was determined using the In Situ Cell Death Detection Kit, TMR red (Roche Molecular Biochemicals), which labels DNA strand breaks (TUNEL assay), followed by the manufacturer’s instructions, followed by flow cytometry analysis. DNA degradation was determined by measuring the orange-red (555–600 mm; FL2) fluorescence in both pEG-FGF-VR1 and pEFhblc-2 cells.

DNA degradation was determined by staining cellular DNA with PI, followed by flow cytometry analysis. Briefly, transfected cells (0.5–1 \times 10\textsuperscript{5}) were fixed and permeabilized in 70% ethanol (–20 °C) for 5 min in order to preserve GFP fluorescence. Cytosolic DNA fragments were then extracted by incubation with DNA extraction buffer (0.2 M Na\textsubscript{2}HPO\textsubscript{4}, 1 mM citric acid, 10 mM). Finally, cells were resuspended in DNA staining solution (PBS; 100 \mu g/ml RNase A, 20 \mu g/ml PI) before analysis by flow cytometry. DNA degradation was determined as the percentage of DNA located in the sub-G0/G1 peak of the cell cycle in both GFP-FGR1- and GFP-FGR1+ cells.

For the determination of DNA fragmentation in oligonucleosomal fragments, DNA ladder was extracted from 10\textsuperscript{5} cells using the Easy-DNA kit (Invitrogen) followed by the instructions provided by the manufacturer. Half of the DNA obtained was loaded on 1.5% agarose gel and electrophoresed.

PS exposure was evaluated by staining the cells with annexin V-PE (BD Pharmingen) and 7-aminoactinomycin D (7-AAD; Sigma) following the manufacturer’s instructions. Cells were incubated with 0.5 \mu M of each reagent for 5 min with annexin V-PE in the appropriate binding buffer. Cells were then incubated for 5 min with 7-AAD (1 \mu g/ml) and analyzed by flow cytometry. GFP-FGR1- cells and GFP-FGR1+ cells were electronically gated, and PS (phosphatidylserine) exposure and 7-AAD permeability were analyzed.
in both populations. For continuous recording of annexin-V binding, cells were incubated with Hank’s balanced salt solution supplemented with up to 2.5 mM CaCl₂, and annexin-V PE binding was measured in gated cell populations by the increase in FL2 fluorescence.

**Western Blot Analysis**—Total cellular protein was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Hybond™ ECL™, Amersham Biosciences). Membranes were blocked overnight with 5% nonfat dry milk in PBS buffer. Subsequent antibody incubations and membrane washes were performed in 0.5% nonfat dry milk in PBS, 0.2% Tween 20. The blot was developed with peroxidase-conjugated anti-rabbit or anti-mouse antibodies using ECL reagents.

**Analysis of Changes in ΔΨm**—Changes in ΔΨm were determined by staining the cells after transfection with 100 nM MitoTracker Red (CMX-Ros; Molecular Probes) for 15 min at 37°C in a humidified atmosphere containing 5% CO₂. Cells were then washed in Hank’s balanced salt solution, supplemented or not with calcium (2 mM) when required, and analyzed by flow cytometry by measuring the red (605–635 nm; FL3) fluorescence in both GFP-VR1⁻ and GFP-VR1⁺ cells. The decrease in ΔΨm, as seen as a decrease in CMX-Ros fluorescence. Control experiments were performed using CCCP (10 μM; Sigma). For the continuous recording of measurements determining ΔΨm, changes, cell suspensions were kept at 37°C during data acquisition (20 min).

**Cytochrome c and AIF Release**—Release of cytochrome c and AIF from the mitochondria to the cytosol in sorted GFP-VR1⁻ and GFP VR1⁺ cells was analyzed by Western blot analysis as previously described (25). Supernatants (cytosolic extracts free of mitochondria) and pellets (particulate fraction that contains mitochondria) from digitonin-permeabilized cells were electrophoresed on a 15% SDS-polyacrylamide gel and then analyzed by WB using anti-cytochrome c antibody (THS2012; BD Pharmingen) or polyclonal anti-AIF antibody as described above. To verify proper separation of the mitochondria, membranes were reprobed with anti-cytochrome oxidase antibody. Protein loading was normalized by using an anti-β-actin monoclonal antibody.

**Immunofluorescence**—Transfected cells (10⁶) were washed in PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, preincubated in 5% bovine serum albumin, and incubated for 1 h with primary antibodies (anti-mitochondria, anti-cytochrome c, anti-AIF) in PBS containing 0.5% bovine serum albumin and 0.1% Triton X-100. Cells were washed three times with the same buffer and incubated for 1 h with goat Fab2 anti-human Cy3, goat Fab2 anti-mouse Cy5, or goat
Fab2 anti-rabbit Cy5 (Jackson Immunoresearch Inc.), respectively. After antibody staining, cells were labeled with 4',6-diamidino-2-phenylindole following standard protocols. Confocal images were captured with Bio-Rad microscope model Radiance 2000 MP with four lasers, argon (488 nm), helium-neon (543 nm), red laser diode (633 nm), and infrared multiphoton (Mira 690–1000 nm), mounted on an Olympus IX70 microscope and using a 60 Plan Apo NA, 1.4 objective. For visualization of 4',6-diamidino-2-phenylindole we used two-photon excitation at 760 nm and emission filter HQ390/70.

Measurement of Reactive Oxygen Species (ROS)/Reactive Nitrogen Species (RNI)—Oxidative/nitrative stress was measured by analyzing DCFH2 oxidation to DCF. To this end, cells transfected with pEF-VR1 plasmid were labeled with 10 μM cell-permeable DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) for 30 min, washed, and challenged for the indicated periods of time with different stimuli. The fluorescence corresponding to the oxidized probe was followed by measuring the green (505–545 nm; FL1) fluorescence in the annexin V-PE population (555–600 nm; FL2).

RESULTS

Expression of GFP-VR1 Fusion Protein—We constructed an N-terminal GFP fusion protein (Fig. 1A) that enabled us to simultaneously follow the expression of VR1 (by GFP fluorescence) and the functional consequences of VR1 stimulation with capsaicin in transient expression experiments with J-HM1–2.2 cells. The GFP-VR1 cells in this heterogeneous cell population constituted an excellent internal control for the functional experiments. In routine experiments, GFP-VR1 expression was achieved in 20–40% of Jurkat cells, as evidenced by flow cytometry (Fig. 1B). Expression of the construct was also confirmed by Western blot analysis (Fig. 1C), and its localization to the plasma membrane was assessed by confocal microscopy (Fig. 1D).

Functional Properties of the VR1 Channel in Jurkat Cells—To characterize calcium transport through the VR1 channel, we followed the Ca2+ influx induced by capsaicin by single cell ratiometric calcium imaging in cells loaded with a fluorescent Ca2+ probe (fura 4F, Kd for Ca2+ = 0.77 μM), which allows for an accurate estimation of [Ca2+]i in the micromolar range. Cells bound to coverslips were superfused with the appropriate solutions, and fluorescence emission was imaged for [Ca2+]i quantitation (see “Experimental Procedures”). Individual cells expressing GFP-VR1 were identified by their fluo-
Fig. 4. Calcium influx-induced PS exposure requires extracellular calcium and involves mitochondrial PTP opening. A, J-HM1–2.2 cells expressing GFP-VR1 were treated for 1 h with capsaicin (0.1 μM) in the absence or presence of calcium (2 mM) in the culture medium and CSA (10 μM). B, cells were cotransfected with 10 μg of pEFGFP-VR1 construct and either 30 μg of pEF empty vector (control) or an equivalent amount of the pEF-hbcl-2 expression vector (hbcl-2). hbcl-2 expression was analyzed by WB analysis in the upper panel. Transfected cells were stimulated with 0.1 μM capsaicin and carbachol induced changes in [Ca^2+]. The traces have been averaged for all of the cells in each group, identified by fluorescence at the end of each experiment, and Ca^{2+} influx in GFP-VR1+ and GFP-VR1− cells present in the same field was analyzed separately. Sequential stimulation of cells with capsaicin and carbachol induced changes in [Ca^{2+}]; the time course of this response is shown in Fig. 2A. The traces have been averaged for all of the cells in each group, identified by GFP fluorescence. Stimulation with capsaicin (0.1 μM) in Ca^{2+}-containing medium induced a fast increase of [Ca^{2+}], from 0.1 to 6 μM in a subpopulation of the cells, which quickly returned to base line after the removal of capsaicin. The capsaicin-sensitive cells corresponded to cells expressing GFP-VR1, as assessed by superimposition of fluorescence images excited at 490 nm (to visualize GFP) and the ratiometric Ca^{2+} images (fura-4F, excited at 340 and 380 nm; see upper panels in Fig. 2A).

We compared Ca^{2+} influx through VR1 with receptor-controlled, capacitative calcium influx. To this end, we took advantage of the fact that J-HM1–2.2 cells stably express HM1R, and HM1R stimulation by carbachol induces inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from the ER and subsequent capacitative calcium influx (20). More than 95% of the cells responded to carbachol with a rise in [Ca^{2+}], of up to 3–4 μM (Fig. 2A and Supplemental Material). We also confirmed the source of the calcium responsible for these changes by stimulating the cells with the agonists in Ca^{2+}-free medium. Under these conditions, the ability of capsaicin to induce the [Ca^{2+}] rise was completely blocked, but carbachol still induced a considerable [Ca^{2+}] increase (Fig. 2A and Supplemental Material). These results indicate unambiguously that the mechanisms for the [Ca^{2+}] increases induced by both agonists are completely different. Capsaicin exclusively induced ligand-gated Ca^{2+} influx, whereas carbachol induced Ca^{2+} release from the ER and subsequent capacitative Ca^{2+} influx. When cells were stimulated for a sustained period of time (5–6 min), [Ca^{2+}], remained increased during the full stimulation period with capsaicin but decreased rapidly from 3–4 to 0.7–0.9 μM within 2 min during stimulation with carbachol (Fig. 2, B and C). This behavior is consistent with different mechanisms of action, since release of Ca^{2+} from the ER by carbachol ceases once the calcium store is emptied, and then the [Ca^{2+}], increase is sustained only by the stimulated capacitative Ca^{2+} influx, which is much smaller than Ca^{2+} influx through VR1. The shapes of the frequency distributions of the maximal [Ca^{2+}], peaks induced by capsaicin and carbachol were completely different (Fig. 2C). The VR1 agonist increased [Ca^{2+}] in only 20–40% of the cells (the ones expressing the vanilloid receptor), and 88% of the peak values were within the 5–7 μM range (and 100% over 3 μM). On the contrary, the HM1R agonist increased [Ca^{2+}] in 95% of the cells, and the frequency distribution was quite uniform in the 1–7 μM range. The fraction of the total cell population that responded with the largest Ca^{2+} peaks (6–7 μM) was quite similar for both agonists (19% for capsaicin and 17% for carbachol; Fig. 2C, right), but the responses to carbachol were transient, whereas the responses to capsaicin were sustained over time (Fig. 2C, left).
Capsaicin Treatment Induces Paraptosis in GFP-VR1⁺ Cells—Once the functional expression of the VR1 was observed, we analyzed whether capsaicin-induced opening of the calcium channel could induce the exposure of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, one feature of cell death. To this end, we electronically gated on GFP-VR1⁺ cells and analyzed annexin V binding by two-color flow cytometry. As shown in Fig. 3A, treatment with capsaicin induced early (15-min) translocation of PS in GFP-VR1⁺ cells. PS exposure increased in a time- and dose-dependent manner (Fig. 3B). After 1 h of treatment with capsaicin, ~70% of the GFP-VR1⁺ cells had PS on the outer leaflet of the plasma membrane (Fig. 3A). As an internal control, we examined the ability of capsaicin to induce PS exposure in the GFP-VR1⁻ cells. Capsaicin did not induce PS exposure in this cell group. Although carbachol induced a [Ca²⁺]₀ increase peaking at 6–7 μM in 17% of the cells (about the same percentage as capsaicin; see above), PS exposure did not increase above background in carbachol-stimulated cells (Fig. 3, A and left panel in B). This observation indicates that a sustained [Ca²⁺]₀ increase, such as the one produced by capsaicin, rather than a high transient [Ca²⁺]₀, is required to trigger PS exposure.

PS exposure not only occurs in apoptosis but also in necrosis. Necrotic cell death is characterized by simultaneous annexin V staining and increased plasma membrane permeability to small solutes such as 7-AAD. To distinguish between apoptotic and necrotic cell death, simultaneous tricolor flow cytometry analysis was performed. As seen in Fig. 3C, GFP-VR1⁺ annexin V⁺ cells excluded 7-AAD during the first h of treatment with capsaicin. This supports the hypothesis that VR1⁺ cells underwent apoptotic rather than necrotic cell death upon capsaicin treatment. GFP-VR1⁺ annexin V⁺ cells became permeable to 7-AAD after 2 h of culture with capsaicin (Fig. 3B, right panel), which demonstrates that these cells ultimately underwent necrotic cell death.

To gain insight into the pathways that connect calcium influx with cell death, we determined the different requirements for triggering PS exposure. First, we tested whether the presence of extracellular calcium was required for the process. In the absence of extracellular calcium (nominally calcium-free culture medium), capsaicin-induced PS exposure was inhibited (Fig. 4A). Therefore, calcium influx through the VR1 channel was required to induce cell death. Second, we investigated the contribution of mitochondria to PS exposure. Cyclosporin A (CSA) has been shown to inhibit apoptotic and necrotic cell death induced by mitochondrial damage by preventing the opening of the PTP (1). We therefore analyzed the effect of CSA on capsaicin-induced PS exposure. As shown in Fig. 4A, CSA prevented PS exposure on the outer leaflet of the cell membrane, suggesting that mitochondrial PTP opening is involved in cell death induced by capsaicin. Overexpression of the anti-apoptotic protein Bcl-2, which protects mitochondria from diverse insults (1), also reduced capsaicin-induced PS exposure, which supports the contribution of mitochondria to the observed cell death (Fig. 4B).

We also analyzed whether caspase activation was involved in this process. Although the general caspase inhibitor Z-VAD prevented anti-Fas-induced apoptosis (measured as annexin-V binding), Z-VAD treatment of GFP-VR1⁺ cells did not interfere with capsaicin-induced cell death (Fig. 5A). Furthermore, GFP-VR1⁺ cells stably expressing the general caspase inhibitor p35 from baculovirus exhibited reduced PS exposure in response to α-Fas but not in response to capsaicin (Fig. 5B). Taken together, these results indicate that caspases are not involved in capsaicin-induced cell death. Additional experiments were performed to test whether capsaicin treatment could also induce other apoptotic events such as cell shrinkage and increase in cell complexity. Treatment with capsaicin induced cell shrinkage (measured by a decrease in forward scatter) and increased cell complexity (increase in side scatter) as assessed by flow cytometry in GFP-VR1⁺ cells (not shown). Z-VAD-insensitive, CSA-inhibitable PS exposure, cell shrinkage, and increase in cell complexity were also induced by capsaicin in three other cell lines transiently expressing GFP-VR1: NCB20 (neuroblastoma), HEK 293 (human embryonic kidney cells), and WEHI231 (pre-B cell line), which indicates that this process is not restricted to Jurkat cells (results not shown).

Recent results suggest that the distinction between apoptosis and necrosis is less clear than initially thought, and caspase-independent programmed cell death and “paraptotic” cell death have been described, which display mixed features of apoptosis and necrosis (26, 27). To distinguish among these possibilities, we performed other classical protocols for the analysis of apoptosis such as caspase-3 activation assays, DNA fragmentation, and TUNEL. These assays were carried out by two-color flow cytometry analysis on electronically gated GFP-VR1⁺ cells or by sorting of GFP-VR1⁺ cells. The flow cytometry experiments quantified (i) active caspase-3 (Fig. 6A), (ii) cells in the sub-G₀/G₁ peak of the cell cycle (Fig. 6B), and (iii) TUNEL⁺ cells. GFP-VR1⁺, capsaicin-treated cells did not express active caspase-3 (Fig. 6A), did not undergo DNA degradation into small fragments such as those observed when α-Fas was used (Fig. 6B), and were TUNEL⁺ (not shown). Consistent with the results of the cell cycle analysis, GFP-VR1⁺-sorted cells treated with capsaicin did not undergo oligonucleosomal DNA laddering as assessed by agarose gel electrophoresis of genomic DNA (Fig. 6C, left). Pulse field electrophoresis of DNA from these cells demonstrated the progressive appearance of 1000-kbp DNA fragments (Fig. 6C, right) that did not progress to 50 kbp.
and/or oligonucleosomal fragmentations as occurs in AIF-dependent apoptosis or in classical apoptosis (26–28). In contrast, treatment with staurosporin, a well-known apoptosis inducer, produced initial fragmentation of the DNA in 1000 kbp and subsequent processing to 50 kbp (Fig. 6C, right). Taken together, the data show that capsaicin-induced cell death fulfills several criteria for both apoptosis and necrosis.

Capsaicin Treatment Induces Caspase-independent \( \Delta \Psi_m \) Dissipation—Protection by CSA and Bcl-2 suggested the involvement of mitochondrial PTP opening in capsaicin-induced cell death (Figs. 4A and 5). To further investigate the role of mitochondria, we labeled cells with CMX-Ros (29) and measured \( \Delta \Psi_m \) by two-color flow cytometry. Treatment with capsaicin induced a decrease of CMX-Ros fluorescence (depolarization) in GFP-VR1\(^+\) cells but not in GFP-VR1\(^-\) cells (Fig. 7A). The decrease in CMX-Ros fluorescence induced by capsaicin was comparable with the one induced by treatment with the protonophore CCCP, a mitochondrial uncoupler that collapses \( \Delta \Psi_m \) (Fig. 7, B and C). To circumvent the possibility that CMX-Ros may be retained into the mitochondria upon depolarization, which could undervalue the \( \Delta \Psi_m \) loss, we performed some experiments in which cells were treated with capsaicin before the staining with CMX-Ros. The uptake of CMX-Ros in GFP-VR1\(^+\) cells pretreated with capsaicin was also decreased in comparison with untreated GFP-VR1\(^+\) (Fig. 7, B, top). In addition, we performed experiments prelabeling cells with a reversible, potential-sensitive dye, tetramethyl rhodamine methyl ester (TMRE). We observed a rapid decrease in the TMRE fluorescence of GFP-VR1\(^+\) cells upon the addition of capsaicin or CCCP, confirming the data obtained with CMX-Ros (Fig. 7B, bottom). The capsaicin-induced mitochondrial depolarization occurred rapidly; \( \Delta \Psi_m \) dissipation was evident after 2–3 min and reached a plateau in 10–15 min (Fig. 7A). Consistent with the annexin V binding results (see above), removal of extracellular calcium or the addition of the PTP inhibitor CSA blocked the ability of capsaicin to dissipate \( \Delta \Psi_m \) (Fig. 7B, A and C). These results suggest that dissipation of \( \Delta \Psi_m \) was not only due to mitochondrial calcium uptake but most probably was a consequence of PTP opening induced by mitochondrial calcium overload. The fact that HM1R triggering did not induce such a \( \Delta \Psi_m \) loss (not shown) provides further support for this hypothesis.

Pretreatment of the VR1-expressing cells with the general caspase inhibitor Z-VAD did not prevent the \( \Delta \Psi_m \) collapse

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**Fig. 6.** \( \text{Ca}^{2+} \) influx does not induce caspase-3 activation or oligonucleosomal/50-kbp DNA fragmentation. A, two-color flow cytometry analysis of gated GFP-VR1\(^+\) cells was performed for detection of active caspase-3. B, cell cycle analysis on gated GFP-VR1\(^+\) cells was performed by DNA staining with PI. C, oligonucleosomal DNA fragmentation (left) and pulse-field agarose (right) gel electrophoresis of genomic DNA from sorted GFP-VR1\(^+\) cells, treated for the indicated time points with capsaicin (0.1 \( \mu \)M), staurosporin (\( \text{Sts}; 2 \mu \)M), or \( \alpha \)-Fas (100 ng/ml) as positive controls.
induced by capsaicin (Fig. 7D), suggesting that caspases do not contribute to the observed ∆Ψm dissipation. In summary, these results indicate that the cell death triggered by capsaicin is mediated by calcium-induced mitochondrial damage but does not involve caspase activation.

**Calcium Influx into Mitochondria Is Necessary for Capsaicin-induced Cell Death**—PTP opening can be triggered by the increase of [Ca2+] inside the mitochondrial matrix (1). The results described so far suggest that the increase of [Ca2+]1 promoted by VR1 activation stimulates mitochondrial Ca2+ uptake through the MCU, leading to an increase in intramitochondrial [Ca2+]1 and subsequent PTP opening. If this hypothesis was correct, depolarization of mitochondria before activation of VR1 should inhibit capsaicin-induced cell death, since ∆Ψm is the driving force for mitochondrial Ca2+ uptake (7, 30, 31). To test this possibility, we studied the effect of capsaicin when mitochondria had been depolarized by prior treatment with CCCP. Fig. 8A shows that pretreatment with CCCP at two different doses prevented PS exposure induced by capsaicin. In another series of experiments, the MCU was blocked by treatment with ruthenium red during the electroporation of cells (32). This treatment also prevented capsaicin-induced PS exposure (Fig. 8B). Thus, inhibition of mitochondrial Ca2+ uptake, either by collapsing ∆Ψm with CCCP or by inhibiting MCU with ruthenium red, prevented cell death. These results indicate that accumulation of Ca2+ into mitochondria is essential for PTP activation and cell death induced by VR1 opening.

**Calcium Overload Does Not Induce Cytochrome c or AIF Release from Mitochondria**—Proapoptotic factors such as cytochrome c and AIF are released from mitochondria during apoptotic processes. Since we have demonstrated that mitochondrial damage is essential for Ca2+-induced cell death, we examined whether cytochrome c and AIF were released from mitochondria in the cell death model studied here, which showed a paraptotic phenotype. Cytochrome c release was assessed by Western blot analysis (Fig. 9A) of soluble and particulate fractions from GFP-VR1+ and GFP-VR1− sorted cells and by immunofluorescence analysis (Fig. 9B) using anti-cytochrome c antibody. Actinomycin D was used as a positive control (33). Sorted GFP-VR1+ cells underwent massive PS exposure (82% annexin-V+ cells; not shown) within 45 min after the capsaicin addition. However, this treatment did not induce detectable cytochrome c release (Fig. 9A). In contrast, treatment with actinomycin D (Act. D) for 4 h induced a lower extent of PS exposure (60% annexin-V+ cells; not shown), but cytochrome c was released from mitochondria in both GFP-VR1+.
and GFP-VR1^−/H11001^− cells as assessed by Western blot analysis of cytosolic fractions (Fig. 9A) and confocal immunofluorescence (Fig. 9B).

We also analyzed whether AIF release from mitochondria was associated with calcium-induced cell death. We could not detect AIF in either the soluble fractions (Western blot; Fig. 9C) or cell nuclei (Fig. 9D) from GFP-VR1^−/H11001^− cells treated with capsaicin. In contrast, staurosporin treatment induced AIF release into the cytosol (Fig. 9C), subsequent translocation to the nucleus, and chromatin condensation (Fig. 9D).

**Calcium Overload Induces Oxidative/Nitrative Stress-dependent Cell Death—** Oxidative/nitrative stress plays a pivotal role in regulating programmed cell death. ROS as well as RNI can be generated after mitochondrial damage and may subsequently mediate apoptosis or necrosis (1). In order to assess free radical production by calcium overload, we analyzed DCFH_2 oxidation in cells expressing the VR1 channel. Capsaicin induced early (5 min) accumulation of DCF as measured by an increase in fluorescence (Fig. 10A) in the annexin V^+ population; after 30 min, the increase in DCF fluorescence was comparable with that of cells treated with the uncoupler CCCP. Antioxidants such as N-acetyl-1-cysteine (NAC) are useful tools in determining the involvement of ROS/RNI in programmed cell death (34). To this end, we measured real time PS exposure in response to capsaicin, in the presence or absence of NAC. As shown in Fig. 10B, NAC inhibited capsaicin-induced PS exposure, which demonstrates the contribution of oxidative and/or nitrative stress to Ca^{2+} influx-induced cell death.

**DISCUSSION**

We describe a unique model of rapid cell death, triggered by Ca^{2+} influx through a receptor-operated channel and involving mitochondrial damage. This cell death fulfills several criteria of apoptosis, such as collapse of Δψ_m, decrease in cell size, increase in cell complexity, exposure of phosphatidylserine on the outer leaflet of the plasma membrane, and lack of permeability to small solutes (7-AAD). Nonetheless, it occurred in the absence of detectable AIF and cytochrome c release, caspase activation, or 50-kbp/oligonucleosomal DNA degradation. Thus, the calcium-induced cell death described in this paper resembles the so-called *paraptotic* programmed cell death, which displays mixed features of both apoptotic and necrotic death (26, 27).

Cell death associated with excitotoxic or ischemic neural lesions and ischemia/reperfusion damage in several tissues shows a paraptotic phenotype (26, 35, 36). It has been proposed that mitochondria play a prominent role in excitotoxic cell death (4, 37), but this model is too complex to determine the specific role of Ca^{2+} overload in cell death (5) (see Introduction). In our model, cell death is triggered exclusively by Ca^{2+} influx through the plasma membrane by the direct and specific
activation of a receptor-operated plasma membrane channel, VR1. The current study provides evidence that mitochondrial Ca\textsuperscript{2+}/H\textsuperscript{1001} overload plays a decisive role in this paraptotic cell death, as shown by the mitochondrial collapse upon VR1 receptor activation leading to PTP opening and cell death, as expected from the electrogentic Ca\textsuperscript{2+}/H\textsuperscript{1001} uptake through MCU and activation of PTP.

It is remarkable that death is prevented by blocking MCU with ruthenium red (Fig. 8B). Consistent with calcium’s role in cell death, removal of extracellular Ca\textsuperscript{2+}/H\textsuperscript{1001} prevented mitochondrial depolarization (Fig. 7A) and cell death (Fig. 4A). Also, CSA, a PTP blocker (1), prevented both the mitochondrial collapse and the PS externalization induced by capsaicin (Figs. 7B and 4A, respectively). In addition, depolarization of mitochondria with CCCP, a mitochondrial Ca\textsuperscript{2+}/H\textsuperscript{1001} uptake inhibitor (7, 31), before VR1 activation prevents the early events of cell death (Fig. 8A). These findings demonstrate that sustained [Ca\textsuperscript{2+}/H\textsuperscript{1001}]\textsubscript{c} levels in the low micromolar range are sufficient to trigger PTP opening, mitochondrial damage, and cell death.

Stimulation of T cells expressing HM1R with carbachol triggers AICD (12), but the cell death induced by carbachol is much slower than the one induced by VR1 stimulation (Fig. 3A; compare with Ref. 12). Direct comparison of the effects of capsaicin and carbachol on [Ca\textsuperscript{2+}/H\textsuperscript{1001}], showed important differences (Fig. 2). The effect of the VR1 agonist was much more sustained over time compared with carbachol, as expected from the different mechanisms of action. Whereas capsaicin treatment sustains Ca\textsuperscript{2+} influx during the entire stimulation period, the effect of carbachol is transient, consistent with cessation of Ca\textsuperscript{2+} release once the ER empties. Subsequently, the plasma membrane Ca\textsuperscript{2+}/H\textsuperscript{1001} ATPase pumps out the Ca\textsuperscript{2+} load, and [Ca\textsuperscript{2+}/H\textsuperscript{1001}]\textsubscript{c} quickly returns toward lower levels. At this stage, [Ca\textsuperscript{2+}/H\textsuperscript{1001}], is still maintained moderately high (<1 μM) (Fig. 2B) by Ca\textsuperscript{2+}/H\textsuperscript{1001}.
influx through capacitative mechanisms (calcium release-activated channels), which are activated by the emptying of the ER (15, 16, 38). In response to these, [Ca\textsuperscript{2+}], calcium release-activated channels are partially inactivated, since [Ca\textsuperscript{2+}], is kept above resting levels (39). However, regarding mitochondrial Ca\textsuperscript{2+} accumulation, [Ca\textsuperscript{2+}], below 1 \mu M, the level maintained by carbachol, produces slow mitochondrial Ca\textsuperscript{2+} uptake (7), whereas a [Ca\textsuperscript{2+}], of 7 \mu M, which is sustained with capsaicin treatment, produces a fast mitochondrial uptake. The increase in [Ca\textsuperscript{2+}], from 1 to 7 \mu M accelerated mitochondrial uptake more than 30 times in chromaffin cells (7). Our results show that the mitochondrial calcium overload resulting from capsaicin treatment induced PS externalization within 15 min (Fig. 3A), whereas 1-h treatment with carbachol had no significant effect on this parameter (Fig. 3A). Such an early commitment to death suggests that an irreversible event, most likely PTP opening, has occurred by this time. This hypothesis is supported by the observation that the maximal increase in \Delta\psi, has also taken place after 15 min of treatment (Fig. 7A). In contrast, commitment to apoptosis during AICD only takes place after 3–4 h, when enough FasL protein has been synthesized (12).

Release of proapoptotic factors cytochrome c and AIF from mitochondria occurs during apoptosis. Cytochrome c release may eventually lead to the assembly of the cytochrome c, ATP, Apaf-1, caspase-9, and caspase-3 complex (apoptosome), which drives activation of the caspase cascade (40) and oligonucleosomal DNA fragmentation (41). In contrast, released AIF translocates to the nucleus and produces 50-kbp DNA fragmentation (1). Under conditions of severe ATP depletion and/or inefficient cytochrome c release, it is thought that caspase activation is not achieved, and cells fail to manifest some of the caspase-dependent features of apoptosis, this favoring the adoption of a more “necrotic” phenotype (1, 42). In our model, capsaicin-induced cell death occurred without release of AIF or cytochrome c from mitochondria (Fig. 9). Also calcium-induced mitochondrial damage and cell death occurred without concomitant caspase-3 activation and were not inhibited by general caspase inhibitors. In addition, neither 50 kbp nor oligonucleosomal DNA degradation were found. The lack of AIF release could be associated with an absence of 50-kbp DNA fragmentation, which is one hallmark of AIF-induced programmed cell death (42). Capsaicin-induced cell death was instead associated with cleavage of the DNA into ~1000-kbp fragments (Fig. 6C). Interestingly, such a DNA degradation pattern has been reported during cell death induced by glutamate in cerebellar granule cells (43). This pattern is different from the features of AICD and resembles characteristics reported for caspase-independent cell death (3, 26) and the so-called paraptotic programme (42), cell death (26, 27).

We observed rapid DCDFH\textsubscript{O} oxidation upon VR1 stimulation, and cell death was inhibited by the antioxidant NAC, which supports the role of oxidative/nitritive stress in calcium overload-induced paraptosis. Future experiments using specific NO probes and scavengers will help to identify the nature of the molecules involved in the oxidation of DCDFH\textsubscript{O} (ROS and/or NO) and their relative contribution to paraptosis. Our preliminary data demonstrate that \Delta\psi, loss still occurs when ROS accumulation is inhibited with antioxidant NAC, and these results locate oxidative/nitritive stress downstream of calcium-induced mitochondrial damage. Taken together, our data provide the first evidence for a direct involvement of cellular calcium influx on mitochondrial damage, subsequent oxidative/ nitritive stress, and paraptotic cell death. Supporting the physiological importance of our model, it is remarkable that stimulation of the VR1 receptor with anandamide, an endocannabinoid that accumulates during in vivo induced excitotoxic necrosis/apoptosis (44) and in lipopolysaccharide-stimulated lymphocytes (45), induces mitochondrial damage and subsequent neural and immune cell death (46).

Finally, our results indicate that T cells possess all of the mechanisms necessary for coupling mitochondrial Ca\textsuperscript{2+} overload to cell death. The paraptotic cell death resulting from activation of the transfected VR1 receptors resembles ischemia/ reperfusion or excitotoxic damage found in excitable tissues, which possess mechanisms for massive Ca\textsuperscript{2+} entry. The emerging inference is that the type of stress a cell undergoes determines the cell death response (apoptosis, paraptosis, or necrosis) by evoking the corresponding death mechanisms, which may be universal for all cell types.

Acknowledgments—We thank Dr. J. Alvarez, Dr. J. A. García-Sanz, Dr. M. A. Gijón, Dr. A. Ruiz-Velas, Dr. Á. Van Linden, and Dr. C. Villablanca for critical reading of the manuscript and Dr. Susin for the generous gift of the anti-AIF antibody. We thank E. Olea, M. C. Moreno, I. López-Vidriero, and E. Ruifernández for help with flow cytometry.

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Sustained Calcium Influx Triggers Paraptosis

Calcium Influx through Receptor-operated Channel Induces Mitochondria-triggered Paraptotic Cell Death

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doi: 10.1074/jbc.M211388200 originally published online February 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211388200

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