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Selective induction of apoptosis by capsaicin in transformed cells: the role of reactive oxygen species and calcium

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Abstract

Capsaicin is a vanilloid quinone analog that inhibits the plasma membrane electron transport (PMOR) system and induces apoptosis in transformed cells. Using a cytofluorimetric approach we have determined that capsaicin induces a rapid increase of reactive oxygen species (ROS) followed by a subsequent disruption of the transmembrane mitochondrial potential ($\Delta\Psi_m$) and DNA nuclear loss in transformed cell lines and in mitogen activated human T cells. This apoptotic pathway is biochemically different from the typical one induced by either ceramide or edelfosine where, in our system, the $\Delta\Psi_m$ dissipation precedes the generation of reactive oxygen species. Neither production of ROS nor apoptosis was found in capsaicin-treated resting T cells where the activity of the PMOR system is minimal when compared with mitogen activated or transformed T cells. Capsaicin also induces Ca^{2+} mobilization in activated but not in resting T cells. However, preincubation of cells with BAPTA-AM, which chelate cytosolic free calcium, did not prevent ROS generation or apoptosis induced by capsaicin, suggesting that ROS generation in capsaicin treated cells is not a consequence of calcium signaling and that the apoptotic pathway may be separated from the one that mobilizes calcium. Moreover, we present data for the implication of a possible vanilloid receptor in calcium mobilization, but not in ROS generation. These results provide evidence that the PMOR system may be an interesting target to design antitumoral and anti-inflammatory drugs.

Keywords: apoptosis; mitochondria; ROS generation; calcium; capsaicin

Abbreviations: AM, acetoxymethyl ester; BAPTA: 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid; $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′dihexyloxacarbocyanine iodide; EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; Eth, ethidium; Fluo3, 1-(2-amino-5-[2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl]-phenoxy)-2-(2-amino-5-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid; HE, hydroethidine; PCD, programmed cell death; PI, propidium iodide; PMOR, plasma membrane NADH-oxidoreductase; PT, permeability transition; RFI, relative fluorescence intensity; ROS, reactive oxygen species

Introduction

Eukaryotic cells continuously produce reactive oxygen species (ROS) as side products of redox reactions. Generation of ROS is mostly governed by the mitochondria, and these ROS comprise hydrogen peroxide, hydroxyl radicals and superoxide anions.[1] In addition to the mitochondria, the plasma membrane contains an electron transport chain that appears to be essential in the control of cell growth and differentiation, stimulation of certain transport function and defense against bacteria.[2,3] An important compound of this system is the plasma membrane NADH-oxidoreductase (PMOR) which transfers electrons from cytoplasmic NADH via CoQ to external electron acceptors[4] such as O_2, ferricyanide, transferrin or ascorbate. It is accepted that the PMOR system plays an important role on the regulation of internal redox equilibrium in response to external stimuli.[2,5] Thus, it has been shown that PMOR inhibitors affect the growth and induce apoptosis in different tumor cell lines.[6–9]

Apoptosis, or programmed cell death (PCD), is a natural form of cell death controlled by a constitutively expressed machinery that induces condensation of nucleoplasm and cytoplasm, blebbing of cytoplasmic membranes, and fragmentation of the cell into apoptotic bodies that are rapidly recognized and eliminated by adjacent cells.[10–12] According to the current understanding, morphological and biochemical alterations in nuclear and chromatin structures of cells that undergo apoptosis are controlled mainly, if not all, by the mitochondria.[13–15] Thus, a breakdown of the transmembrane mitochondrial potential ($\Delta\Psi_m$) is an invariant feature of early apoptosis, which precedes DNA fragmentation,[16,17] regardless the cell type and the apoptotic stimuli.[18–23] $\Delta\Psi_m$ disrupture is mediated by mitochondrial megachannel opening (permeability transition, PT)[24–26] leading to the release of cytochrome-c and the so-called apoptotic inducing factor (AIF), which can mediate nuclear fragmentation.[15,27–29] After the initial $\Delta\Psi_m$ dissipation, cells hyperproduce ROS that may contribute to the apoptotic pathway.[30] It has been shown that these ROS are derived from the complex III of the mitochondrial respiratory chain.[22,30] Nevertheless, it has been demon-

strated that in some other systems, mitochondrial ROS production preceed $\Delta\Psi_m$ dissipation.[31–33]

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), the pungent ingredient in a wide variety of red peppers of the genus *Capsicum*, and other related compounds are collectively referred as vanilloids. These compounds have been shown to interact at specific membrane recognition sites (vanilloid receptors) expressed almost exclusively by primary sensory neurons involved in nociception and neurogenic inflammation (revised in Holzer, 1991[34]), and in tracheobronchial tissues.[35,36] The recent cloning of a vanilloid receptor, termed VR1 (vanilloid receptor subtype 1), has shown that this receptor belongs to the family of putative store-operated calcium channels. The VR1 cDNA encodes a protein of 838 aminoacids with six transmembrane domains, a short hydrophobic stretch located between transmembrane regions five and six, and the amino terminal region contains a proline rich region followed by three ankyrin repeat domains.[37]

The vanilloid receptors may mediate the effects of capsaicin and other vanilloids in some cell systems. Thus, exposure of nociceptor terminals to capsaicin leads to excitation and desensitization in dorsal root ganglion neurons and an increase in intracellular calcium.[37–39] These biological activities underlie the use of capsaicin as an analgesic agent in the treatment of painful disorders such as peripheral neuropathies and rheumatoid arthritis.[40–42] In addition to these biological activities, capsaicin is an inhibitory quinone analog that can inhibit the PMOR system and induce apoptosis by a vanilloid-receptor independent pathway.[7–9] Interestingly, capsaicin seems to inhibit only the PMOR of plasma membrane vesicles from cancer cells and not that of non-tumoral cells[6] suggesting that a possible specific PMOR system is upregulated in transformed cells. In the immune system, capsaicin has been shown to have immunomodulatory effects, as indicated by its ability to modulate lymphocyte proliferation and immunoglobulin production.[43–45]

In the present report we investigated the biochemical pathways activated by capsaicin in transformed cells and in activated human peripheral T cells. We now show that capsaicin induces apoptosis in transformed cells and in mitogen activated peripheral blood cells but not in resting cells. The DNA fragmentation in transformed and activated T cells is preceded by an increase in the concentration of cytosolic free calcium and ROS production. We also present evidence suggesting that the apoptotic effect of capsaicin is not related to the changes in Ca^{2+} and that $\Delta\Psi_m$ breakdown is mediated by oxidative damage of the mitochondria.

Results

Induction of apoptosis by capsaicin and, both ceramide and edelfosine are mediated through different biochemical pathways in transformed cells

It is now accepted that mitochondrial $\Delta\Psi_m$ breakdown and ROS generation are invariant features of early apoptosis.[18–23,33] Thus, we studied the role of the mitochondria and ROS generation in the apoptotic pathway induced by capsaicin (250 μM), C_6-ceramide (25 μM) and edelfosine (5 μg/ml) in three different human tumor cell lines (Jurkat, a T cell line; K562, an erythroleukemic cell line; and 293T a human embryonic kidney-derived cell line expressing the SV40 large T-antigen). The cells were treated for 6 h and ROS generation and $\Delta\Psi_m$ dissipation detected by double staining experiments, using HE (non-fluorescent) that becomes ethidium (Eth, red fluorescent) after its oxidation via ROS, and $DiOC_6(3)$ (green fluorescent), a cationic probe that accumulates into mitochondria as a function of its potential.[46] After 18 h treatment, hypodiploidy (loss of fragmented DNA), as a marker for apoptosis, was also analyzed by PI staining.

As shown in Figure 1, untreated cells presented a high $\Delta\Psi_m$ ($DiOC_6(3)^{high}$) and low levels of intracellular ROS $(HE\rightarrow Eth)^{low}$. These values were taken as background data. As expected, capsaicin induced in all cell lines an increase in the percentages of $DiOC_6(3)^{low}/(HE\rightarrow Eth)^{high}$ and $DiOC_6(3)^{high}/(HE\rightarrow Eth)^{high}$ cells, while the percentage of $DiOC_6(3)^{low}/(HE\rightarrow Eth)^{low}$ cells did not change when compared to untreated cells. That is, ROS are first generated and then $\Delta\Psi_m$ breakdown appears. On the other hand, a different biochemical pathway preceded the apoptosis mediated by either ceramide or edelfosine in Jurkat, K562 and 293T cells. The treated cells become first $DiOC_6(3)^{low}/(HE\rightarrow Eth)^{low}$, and then the intracellular levels of ROS were increased $(HE\rightarrow Eth)^{high}$. This pathway has been previously described and is characteristic of most of apoptotic stimuli.[22,23,30] Regardless of the stimuli, the apparition of $DiOC_6(3)^{low}$ cells correlates with a significant increase in the number of hypodiploic apoptotic cells. Finally, it is interesting to note that K562 cells, which do not undergo apoptosis in response to edelfosine and only partially to ceramide, are still very sensitive to capsaicin treatment.

ROS induced by capsaicin treatment are independent of mitochondrial metabolism

In order to analyze whether ROS are produced in the electron chain of the mitochondria or elsewhere (i.e. the PMOR system), we have treated Jurkat cells in presence or absence of ferricyanide, a cell-impermeant extracellular electron acceptor that potentiates the activity of the PMOR system,[47,48] or rotenone (an inhibitor of the complex I of the mitochondrial electron chain), prior to capsaicin treatment. In Figure 2 it is shown that in Jurkat cells, the preincubation with ferricyanide enhances the generation of intracellular ROS mediated by capsaicin that was also accompanied by an increase in the percentage of $DiOC_6(3)^{low}/(HE\rightarrow Eth)^{high}$ cells. Ferricyanide alone did not change the levels of intracellular ROS. On the other hand, we show that rotenone treated cells are mostly $DiOC_6(3)^{low}$, because a high $\Delta\Psi_m$ is no longer supported as a consequence of the mitochondrial electron chain inhibition. This inhibition at complex I blocks the mitochondrial ROS production, which are generated mainly at complex III.[22,30,32] Nevertheless, treatment with capsaicin still forms ROS, suggesting that they are probably generated outside the mitochondria.

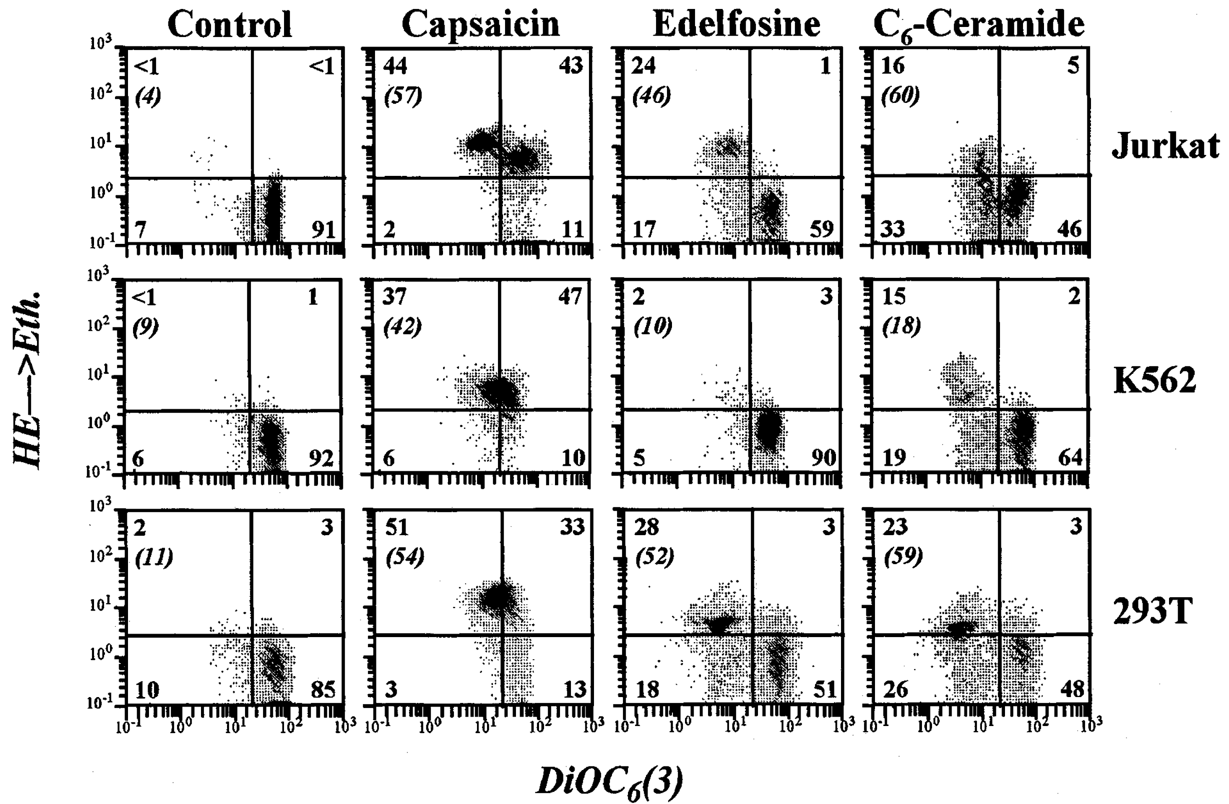

Figure 1 Induction of apoptosis by capsaicin, edelfosine and ceramide in transformed cells. Jurkat, K562 and 293T cells were treated with capsaicin, edelfosine or C_6–ceramide. After 6 h treatment, half of the cells were collected and the simultaneous $\Delta\Psi_m$ disruption and ROS generation detected by cytofluorimetry. The results represent the percentage of cells obtained in biparametric histograms delimited by four compartments, namely, $\Delta\Psi_m^{high}$ (normal cells, bottom-right compartment); $\Delta\Psi_m^{low}$ (bottom-left); $(HE\rightarrow Eth)^{high}$ (ROS generating cells, top-right), and $(HE\rightarrow Eth)^{high}/\Delta\Psi_m^{low}$ (preapoptotic cells, top-left compartment). Apoptosis was measured after 18 h of treatment by cell cycle analysis with PI staining (percentage of apoptotic cells between brackets)

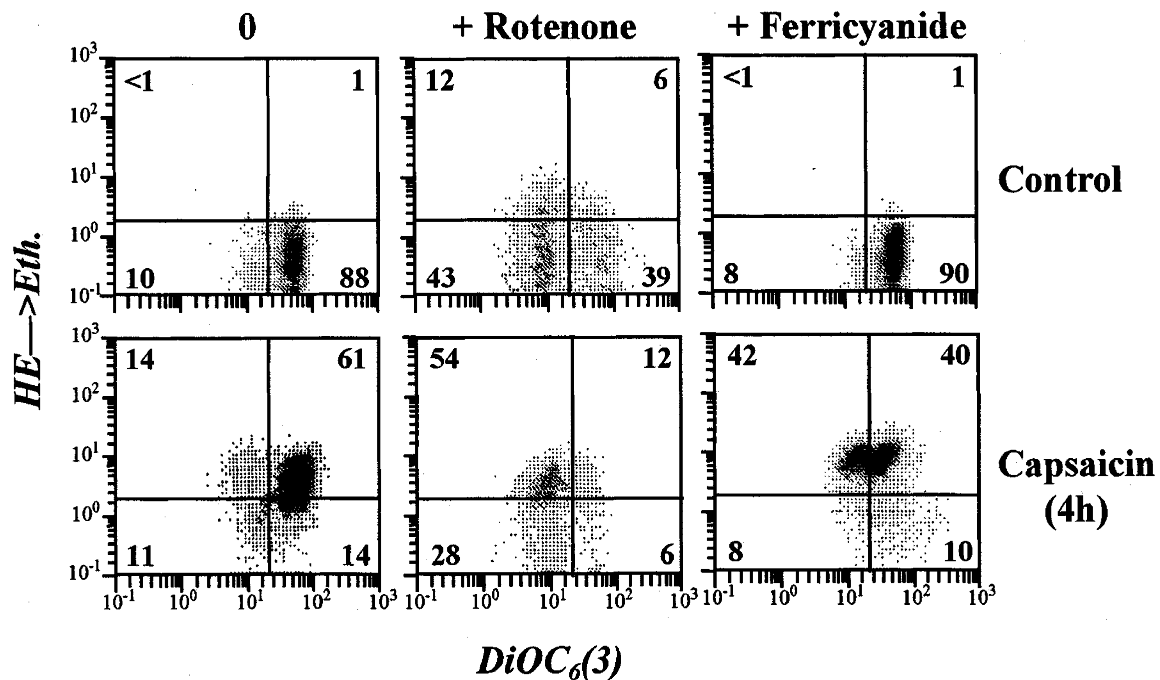

Figure 2 The ROS generation in capsaicin-treated Jurkat cells is mediated by PMOR inhibition. Jurkat cells were preincubated for 1 h with either rotenone (20 mM) or ferricyanide (100 μM), and then incubated with 250 μM capsaicin for 6 h at 37°C. The ROS generation and $\Delta\Psi_m$ disruption were detected as in Figure 1

The PMOR is upregulated in mitogen-activated and transformed T cells

It has been previously reported that undifferentiated cells show a higher PMOR activity than differentiated cells.[6] Thus, we were interested in measuring the activity of the PMOR system in intact T cells under different culture conditions. The activity of the PMOR system can be measured by its specific capacity to reduce ferricyanide or to regenerate ascorbate to its active form.[4,49,50] In Figure 3 both activities are compared in resting peripheral T cells, in mitogen-activated T cells and in the Jurkat transformed cell line. We have detected that the ability to reduce ascorbate was significantly increased in both activated T cells and Jurkat cells, when compared with resting peripheral T cells. Similar results were obtained with the rate of ferricyanide reduction, indicating that the PMOR system measured in intact cells was somehow upregulated by cellular activation.

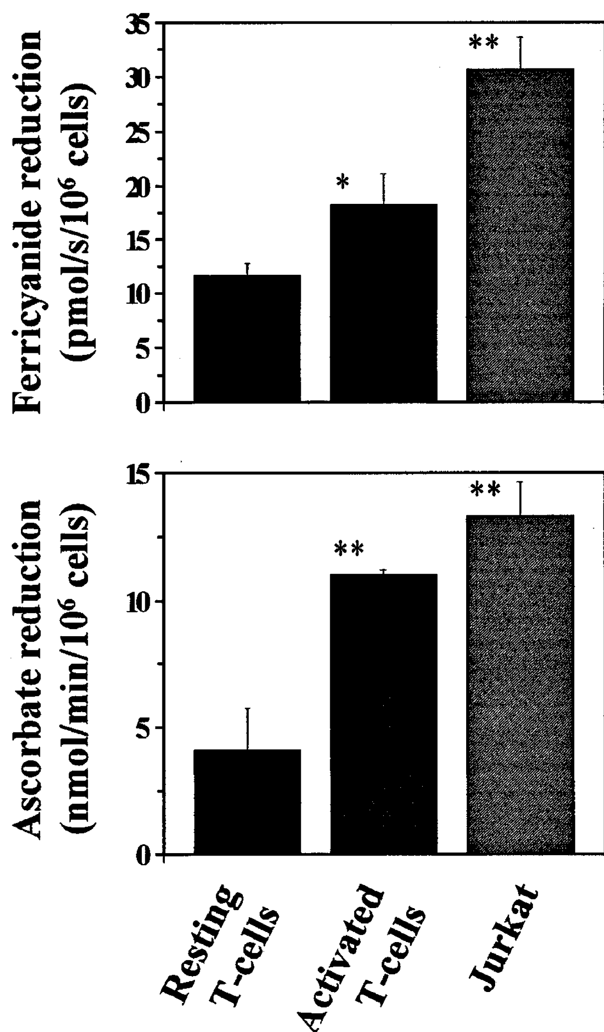

Figure 3 Determination of PMOR activity in resting and mitogen-activated peripheral T cells and Jurkat cells. The ferricyanide (upper panel) and the ascorbate reductions (lower panel) mediated by intact cells were measured as described in Material and Methods. The results represent the mean ± S.E. of three independent experiments. $^*P \leq 0.05$; $^{**}P \leq 0.01$

Capsaicin induces apoptosis in mitogen-activated T cells but not in resting T cells

Our previous results have shown a close relationship between the PMOR system and the apoptotic pathway induced by capsaicin. Since activated T cells upregulate this system when compared with resting cells, we studied the effect of capsaicin in both cells. To address this point, resting and mitogen-activated peripheral T cells were stimulated with capsaicin for 6 h and the ROS generation and the $\Delta\Psi_m$ measured as above. Figure 4A shows that in activated T cells, this vanilloid induced an increase in the levels of intracellular ROS in a high percentage of cells. Moreover, some of these cells showed at this time a disruption of the $\Delta\Psi_m$, which increased after 12 h treatment (data not shown). Thus, activated T cells behave similarly to Jurkat cells, although the latter were more sensitive to capsaicin, a fact which may be explained by its clonality and by its higher activity of the PMOR system (Figure 3). In sharp contrast to activated T cells, neither ROS generation nor $\Delta\Psi_m$ dissipation could be detected in capsaicin-treated resting T cells (Figure 4), even in the presence of ferricyanide (data not shown). As expected, the initiation of the preapoptotic cascade (measured as the $\Delta\Psi_m$ breakdown; i.e. lesser staining with $DiOC_6(3)$), mediated by capsaicin, led to apoptosis in both activated T cells and in Jurkat cells, but not in resting T cells (Figure 4B). Although capsaicin-mediated apoptosis is concentration- and time-dependent,[9] we have not found signs of apoptosis in resting T cells stimulated up to 5 days with 300 μM capsaicin (results not shown).

Capsaicin mobilizes calcium in transformed and activated T cells

There is evidence that PMOR system can regulate the trans-plasma membrane calcium fluxes in synaptosomes[51,52] and that vanilloids increase the uptake of Ca^{2+} in neuronal cells through the vanilloid receptor.[37–39] To study a possible relationship between ROS generation, mediated by capsaicin and the intracellular calcium mobilization, we performed a set of experiments. First, to determine if capsaicin mobilizes calcium in lymphoid cells, we have analyzed the calcium levels in treated and untreated cells by measuring the degree of fluorescence of the Fluo3-AM, which, once inside the cell, captures Ca^{2+} with high affinity and becomes near 70-fold more fluorescent.

Figure 5A shows that capsaicin is able to mobilize calcium in both Jurkat cells, and mitogen-activated peripheral T cells but not in resting T cells. Nevertheless, resting T cells can mobilize intracellular calcium in response to the calcium ionophore A23187. The kinetics of calcium mobilization in capsaicin treated cells show that capsaicin induces a rapid mobilization of calcium, being detectable shortly after 1 min in Jurkat and in activated T cells, with a plateau at 30 min for activated T cells and more than 60 min for Jurkat cells. Again, we did not find a significant calcium mobilization in capsaicin-stimulated resting T cells (Figure 5B). To investigate in more detail this calcium mobilization in Jurkat cells, we preincubated the cells with BAPTA-AM (an intracellular chelator of Ca^{2+})

and in Figure 6 it is shown that capsaicin-induced Fluo3 fluorescence was inhibited up to 62% in the presence of the calcium chelator. Moreover, the presence of EGTA also affects the capsaicin-mediated calcium mobilization (42% reduction). Altogether, these results indicated that both, the extracellular calcium and the intracellular pool of calcium, participate in the calcium mobilization detected in capsaicin-treated T cells.

Different pathways mediate the ROS generation and the calcium mobilization induced by capsaicin

Since kinetics experiments have shown that calcium mobilization precedes the ROS generation (Figure 5B and reference 9), we studied the possibility that an increase in the intracellular calcium was responsible for the ROS generation found in capsaicin-treated Jurkat

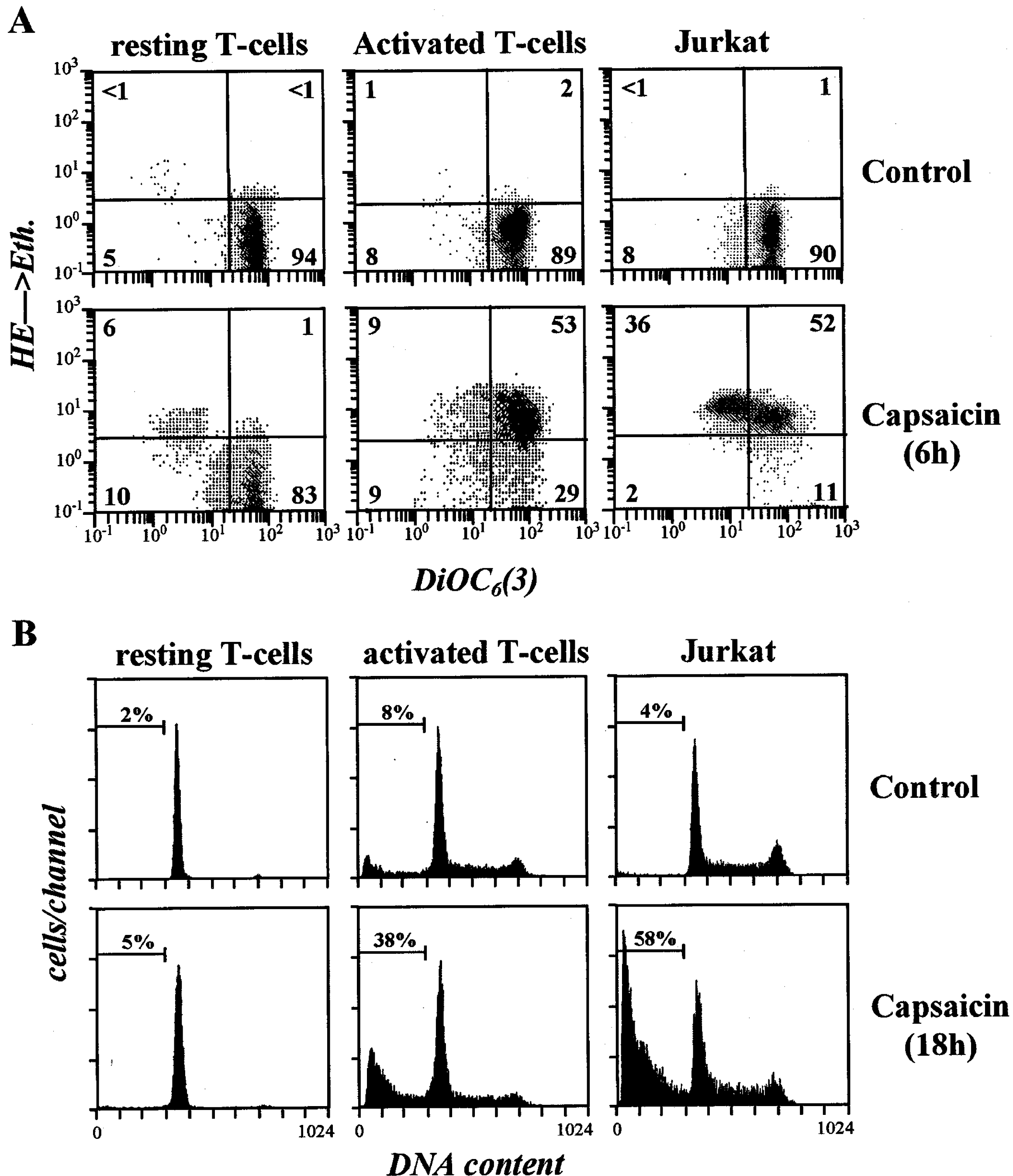

Figure 4 Effects of capsaicin in ROS generation, $\Delta\Psi_m$ dissipation and apoptosis in resting and mitogen-activated T cells. Resting and activated peripheral T cells were incubated for either 6 h (**A**) or 18 h (**B**) in the presence of capsaicin (250 μM). (**A**) Effects of capsaicin in the generation of intracellular ROS and $\Delta\Psi_m$ disruption. (**B**) Determination of apoptosis by cell cycle analysis

cells. To address this point, we preincubated Jurkat cells with BAPTA-AM before treatment with capsaicin. In Figure 7A, we show that calcium chelation did not affect the ROS generation induced by capsaicin but also accelerated the conversion of $DiOC_6(3)^{high}$/$(HE{\rightarrow}Eth)^{high}$ to $DiOC_6(3)^{low}$/$(HE{\rightarrow}Eth)^{high}$ cells. Moreover, to investigate a possible interaction of capsaicin with a vanilloid receptor in Jurkat cells, we preincubated the cells with a tenfold molar excess of vanillate, which contains the same vanillic group that capsaicin. Interestingly, vanillate that did not prevent ROS generation (Figure 7A), was able to inhibit up to 70% of the calcium mobilization induced by capsaicin in Jurkat cells. This inhibition was specific for capsaicin since the mobilization of calcium induced by A23187 was not affected by preincubation with the vanillate. These results strongly suggest that capsaicin activates two separated pathways, namely, a vanilloid receptor-dependent mobilization of calcium and an apoptotic pathway, which is independent of this putative receptor and is mediated by the inhibition of the electron chain of the PMOR system.

Discussion

According to the current understanding, the apoptotic cascade is controlled mainly by the mitochondria,[13–15] through the opening of its megachannel pores (PT), and the release of apoptogenic factors such as AIF and cytochrome-c. At least, there are two general mechanisms to induce PT:[17] the first one, which is mediated by ICE (interleukin-1β converting enzyme, caspase-1), is independent of the protective effect of Bcl-2 (typical of Fas-induced apoptosis). The second mechanism is activated by death factors that mediate a pro-oxidative environment, and is Bcl-2 dependent. Notwithstanding, the mitochondrial release of AIF and cytochrome-c, activates other caspases and enzymes, such as CPP32/caspase-3 or PARP, leading to the morphological and biochemical alterations in nuclear and chromatin structures of cells.[17,53]

We and others have described that vanilloids induce apoptosis in transformed cells.[6–9] Similar to ceramide[30,54,55] and edelfosine,[56] the biochemical pathway activated by capsaicin is Bcl-2 dependent and can be

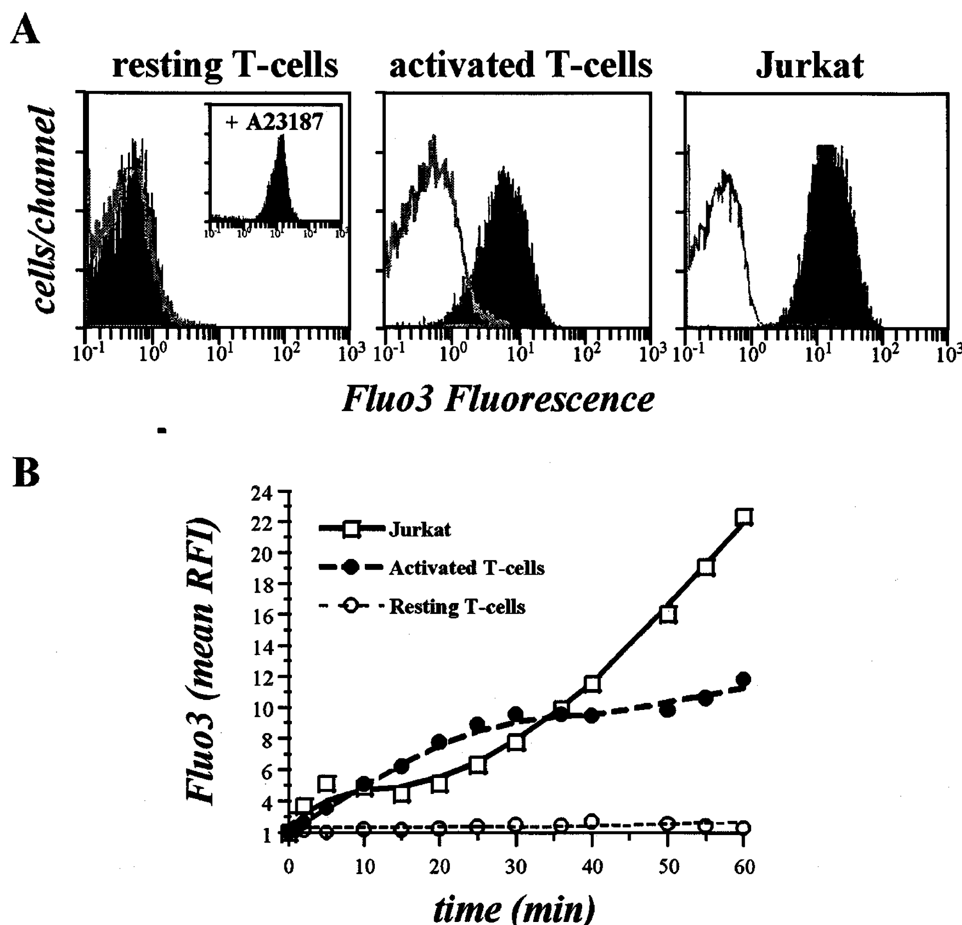

Figure 5 Calcium mobilization induced by capsaicin. Intracellular calcium levels were determined in resting and mitogen-activated T-cells, and in Jurkat cells treated with capsaicin (200 μM). (**A**) Calcium detection after 1 h treatment. Intracellular calcium background is represented as white histograms. Insert in left panel represents the calcium mobilization induced with A23187 after 15 min treatment. (**B**) Kinetics of capsaicin-induced calcium mobilization. The calcium levels were measured by the increase of Fluo3 green fluorescence and the data represents the mean of relative fluorescence intensity (RFI) of Fluo3 for the whole cell population

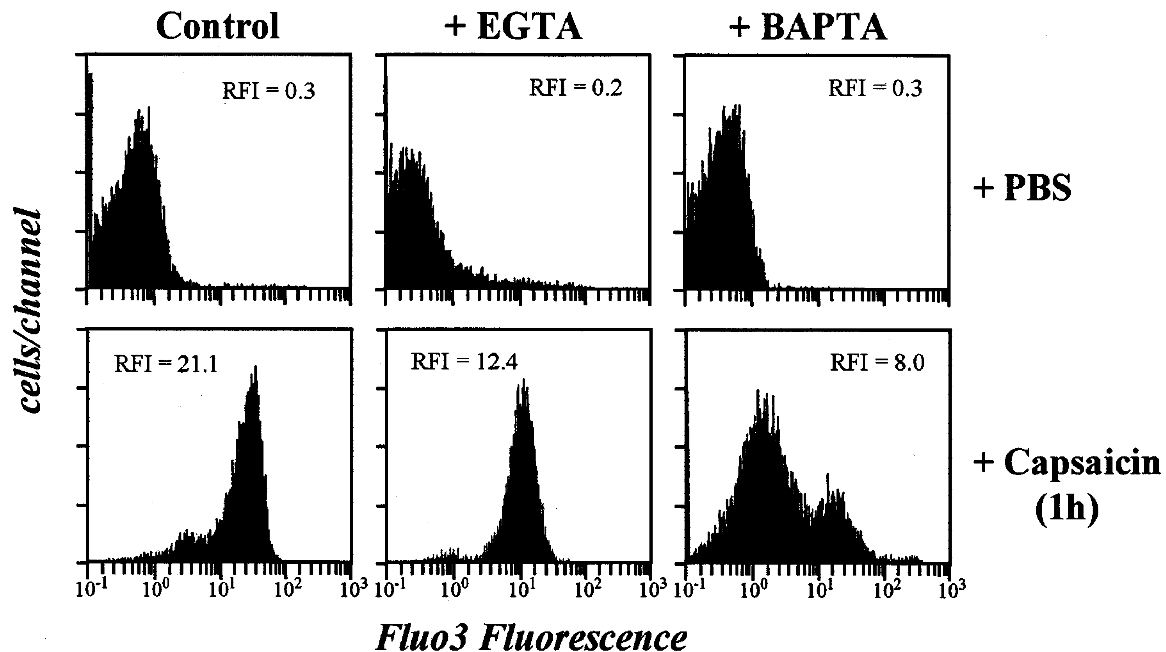

Figure 6 Modulation of calcium mobilization in capsaicin treated Jurkat cells. Cells were stained with Fluo3-AM in calcium free PBS, preincubated with either EGTA or BAPTA-AM for 30 min and then treated with or without 200 μM capsaicin for 1 h in PBS. Numbers indicate the relative fluorescence intensity of Fluo3 for the whole cell population. One representative out of three independent experiments is shown

inhibited by mitochondrial antioxidants that prevent mega-channel opening.[7,9] However, contrary to the ceramide and edelfosine in our cell model, capsaicin treatment is characterized by a hypergeneration of ROS that precedes $\Delta\Psi_m$ disruption. Interestingly, capsaicin has the advantage of its selectivity to hypergenerate ROS and induces apoptosis only in activated and transformed cells. Our results demonstrate that ROS generation is mediated by inhibition of the PMOR system and that they do not come from a mitochondrial source, because inhibition at complex I of the mitochondrial electron chain did not prevent ROS generation. Electrons from the conversion of NADH to NAD^+ are used by the PMOR system to reduce extracellular ferricyanide (as the final electron acceptor, NADH-ferricyanide-reductase activity[47,48]) or to generate H_2O_2 from molecular oxygen (NADH-oxidase activity). Incubation with extracellular acceptors potentiates both activities.[57] Thus, upregulation of the PMOR system with ferricyanide will increase the pool of electrons susceptible to be redirected to form ROS upon a possible blockage of the PMOR electron chain with capsaicin. This would explain our results, in which the addition of ferricyanide to the medium enhances the ROS generation induced by capsaicin. The same result has been obtained using ascorbate to enhance PMOR activity (data not shown).

The capacity to inhibit the PMOR system by capsaicin seems to be related to its vanillic group because related compounds having in common only this structure have the same effect over the electronic chain, as is the case of resiniferatoxin, a capsaicin analog derived from plants of the genus *Euphorbia* that is 5–7-fold more potent that capsaicin.[7,9] The degree of the electron chain inactivation could be related to the hydrophobicity of the vanilloid compound, which is defined by its non-vanillic moiety, as reported by Shimomura *et al*.[58] with purified mitochondrial NADH-oxidase. This explains the higher capacity of resiniferatoxin to induce ROS generation and apoptosis (it has a diterpene group, whereas capsaicin has a single C_9-alkyl chain), and the lack of effect of vanillic acid (Figure 7) that is practically hydrosoluble and therefore cannot enter the cell.

It has been shown by several groups that capsaicin induces intracellular calcium mobilization in some cell types, mainly primary sensory neurons, and that this effect is mediated via interaction with the so-called vanilloid receptor.[37,39,59] This receptor seems to recognize the vanillic group of vanilloid compounds because binding of ^{3}H-resiniferatoxin is inhibited by competition with capsaicin and other analogs.[60] In the present paper we show that capsaicin induces calcium mobilization also in lymphoid cells. But, as for ROS generation, this occurs only in activated or transformed lymphoid cells. In neuronal cells, capsaicin provokes the uptake of external $^{45}Ca^{2+}$, which can be inhibited by ruthenium red (a calcium channel blocker), concluding that in these cells calcium mobilization is mainly of extracellular origin (see revision of Holzer:[34]). However, in lymphoid cells, our results with BAPTA-AM and EGTA have demonstrated that capsaicin mediates the uptake of extracellular calcium and mobilizes the intracellular 'pools' of calcium.

More importantly, as for other types of cells, a cell surface receptor seems to be implicated in calcium mobilization also in activated lymphoid cells because competition experiments with a tenfold excess of vanillic

acid inhibit the calcium mobilization up to 70% in capsaicin treated cells. It is interesting to note that vanillic acid does not affect the capsaicin-mediated PMOR inhibition. As discussed, the vanillic acid is hydrosoluble and can not enter the cells, but it would interact with the vanilloid recognition site of the membrane vanilloid receptor in a similar way to capsazepine, which binds VR1 but does not mobilize intracellular calcium.[37] This could explain the effects of vanillic acid in the calcium mobilization mediated by capsaicin.

Mobilization of intracellular calcium has been also involved in redox destabilization in some apoptotic

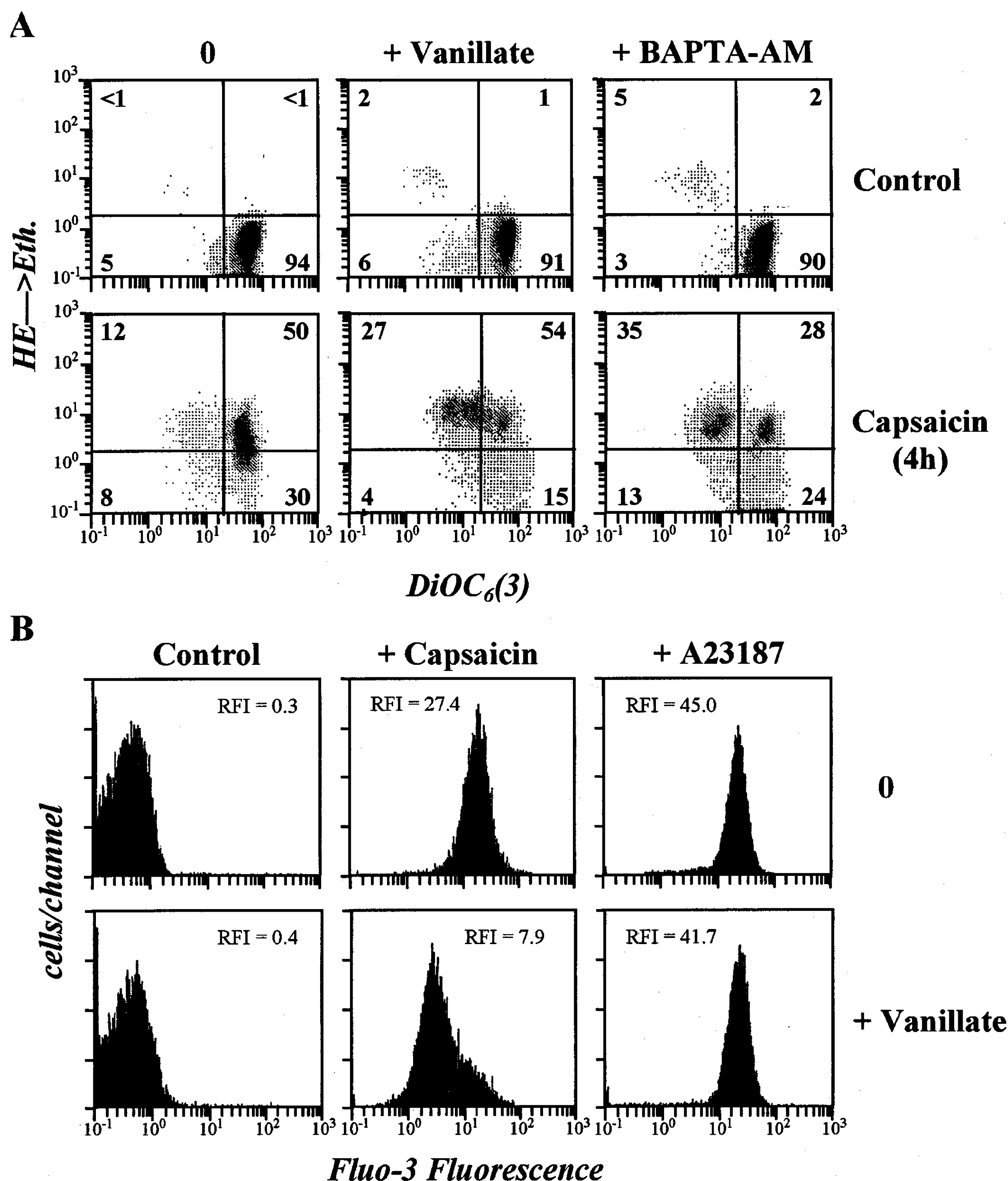

Figure 7 Vanillate modulation of ROS generation and calcium mobilization in capsaicin treated cells. (**A**) ROS generation and $\Delta\Psi_m$ disruption in Jurkat cells. The cells were preincubated with either vanillic acid or BAPTA-AM for 1 h, and treated with 250 μM capsaicin for 4 h. Cells were immediately stained with $DiOC_6(3)$ and HE as described in Figure 1. (**B**) Calcium mobilization analysis. Jurkat cells were stained with Fluo3-AM, preincubated with vanillic acid for 1 h and treated with either 200 μM capsaicin for 1 h or 1 μM A23187 for 15 min. Numbers represent the mean of relative fluorescence intensity of Fluo3 in the whole cell population. One representative out of three independent experiments is shown

pathways. In this case, it has been described that the ROS generated are mainly of mitochondrial origin (by causing mitochondrial PT, that in turn disrupt the $\Delta\Psi_m$ and subsequently could uncouple the mitochondrial electron chain).[30,61] We think that this is not the case of capsaicin induced ROS generation, since we have shown that intracellular ROS are mainly produced out of the mitochondria. It is also possible that ROS are generated as a consequence of a PMOR blockage by increased levels of calcium and not by capsaicin directly. However, we found that the inhibition of calcium by BAPTA-AM or vanillic acid not only was not followed by a reduction in the level of ROS but it increased the amount of intracellular ROS and accelerated the $\Delta\Psi_m$ disruption. As a 'hypothetical model', it is possible that calcium, as well as other divalent cations, could reduce the rate of the turnover-dependent activation of the PMOR NADH-oxidase, as occurs with the mitochondrial NADH-oxidase.[62] This could explain the effects on ROS generation when calcium is removed or its efflux prevented in capsaicin treated Jurkat cells.

Another interesting finding in the present report is the fact that resting peripheral T cells do not undergo apoptosis or mobilize calcium in response to capsaicin. The low levels of PMOR activity found in these cells can explain the lack of ROS generation and apoptosis after capsaicin treatment. The PMOR system seems to be upregulated upon activation. Nevertheless, we cannot exclude the possibility that transformed or, in general, proliferating cells, have a PMOR system sensitive to capsaicin, different from that of quiescent cells, which seem to be insensitive.[6] Since we have shown that calcium mobilization in activated cells is probably mediated through a vanilloid receptor, it is likely that such a receptor is not expressed in resting T cells as has been shown by the vanilloid receptor type 1,[37] but it can be upregulated after mitogen activation. We are currently working on this possibility.

Taken together, these results provided evidence that plasma-membrane electron-transport system may be an interesting target to design capsaicin-like antitumoral and anti-inflammatory drugs, without affecting other properties of vanilloids, such as pain relief caused by nociceptive neuron desensitization.

Material and Methods

Cell lines and reagents

Jurkat and K562 cells (ATCC, Rockville, MD, USA) were maintained in exponential growth in RPMI 1640 medium (Bio-Whittaker, VerViers, Belgium) and the human embryonic kidney-derived 293 cells (ATCC) in DMEM (Bio-Whittaker), both supplemented with 10% heat inactivated FCS, 2 mM L-glutamine and the antibiotics penicillin and streptomycin (Gibco, Paisley, Scotland). BAPTA-AM, Fluo3-AM and the ionophore A23187 were from Molecular Probes Europe (Leiden, The Netherlands). Edelfosine (ET-18-OCH_3) was obtained from R. Berchtold (Biochemisches Labor, Bern, Switzerland). All other reagents not cited above or later were from Sigma Chemical Co. (Barcelona, Spain).

Isolation of peripheral human T cells

Human PBMC, from healthy adult volunteer donors, were isolated by centrifugation of venous blood on Ficoll-hypaque density gradients (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). Macrophages were removed by incubating the PBMC on 100 mm plastic Petri plates at 37°C for 60 min, and the remaining cells were passed twice through a nylon wool column to deplete residual B cells and macrophages. Nylon Wool T cells were usually 95% $CD3^+$ and less than 5% $CD25^+$. To obtain T cell blasts, 2×10^6/ml PBMC were incubated with ConA (5 μg/ml) in complete medium for 72 h and then passed through a Nylon Wool Column to deplete residual B cells. Mitogen-activated T cells were usually more than 80% $CD25^+$.

Cytofluorimetric analysis of mitochondrial transmembrane potential, ROS generation and nuclear DNA loss

To evaluate the mitochondrial transmembrane potential ($\Delta\Psi_m$) and the superoxide anion generation (ROS), cells (10^6/ml) were incubated in PBS with $DiOC_6(3)$ (green fluorescence) (20 nM) (Molecular Probes Europe) and dihydroethidine (HE) (red fluorescent after oxidation) (2 μM) (Sigma) for 20 min at 37°C, followed by analysis on an Epics Profile II Analyzer cytofluorimeter (Coulter, Hialeah, FL, USA). The percentage of cells undergoing chromatinolysis (subdiploid cells) was determined by ethanol fixation (70%, for 24 h at 4°C), followed by RNA digestion (RNAse-A, 50 U/ml) and propidium iodide (PI, 20 μg/ml) staining, and analyzed by cytofluorimetry as previously described.[63]

Determination of PMOR activity

Short-term ascorbate oxidation was carried out on intact Jurkat cells, peripheral resting T cells and mitogen-stimulated T cells, in 100 mM Tris-Cl pH 7.4, with a final volume of 1.0 ml, in the presence of 2 mM potassium cyanide and 3 mM ascorbate. The ascorbate oxidation was followed by a direct reading at 265 nm for 10 min at 37°C either in the presence or absence of cells as described.[64] Specific activity of the ascorbate reduction (regeneration) was calculated by the difference between the rates of chemical ascorbate auto-oxidation with and without cells.[65] An extinction coefficient of 11.2 $mM^{-1}\ cm^{-1}$, determined on the basis of ascorbate absorbance in Tris-HCl buffer, was used in calculations of specific activities.[66] Ferricyanide reduction by cells was measured by absorbance change at 420 minus 500 nm in TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4/25 mM Trizma base pH 7.4), containing 10^5 cells and 0.1 mM ferricyanide. An extinction coefficient of 1 $mM^{-1}\ cm^{-1}$ was used in calculations of specific activies.[4,49]

Determination of cytosolic free calcium

Intracellular calcium levels were measured using Fluo3-AM (final concentration: 1 μM; excitation: 488 nM; emission: 525 nM). The cells (5×10^6 cells/ml) were loaded with the fluorochrome in calcium free PBS for 20 min at 37°C. The loading solution was removed and the cells incubated in DMEM medium (10^6 cells/ml), and treated with or without capsaicin (200 μM) for the indicated times at 37°C. Specific fluorescence was immediately measured on an Epics profile II cytofluorimeter. To chelate cytosolic or extracellular Ca^{2+}, cells were preincubated in PBS for 30 min at 37°C with either BAPTA-AM (5 μM) or EGTA (50 μM), respectively. Additional experiments to control calcium mobilization were made by preincubating cells with either ruthenium red (50 μM) or vanillic acid (2 mM).

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