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Cytotoxicity of capsaicin in monkey kidney cells: lack of antagonistic effects of capsazepine and Ruthenium red

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Abstract Capsaicin is a natural product of *Capsicum* peppers, excitatory effects of which have been shown to be mediated by the recently cloned vanilloid receptor 1 (VR1). Since previous studies have shown that capsaicin inhibits protein synthesis, experiments were performed to investigate whether this effect is mediated by VR1 receptor on cultured monkey kidney cells (Vero cells). The capsaicin uptake was assessed in cellular homogenate and in medium by high-performance liquid chromatography (HPLC) separation and quantification on C18 reverse-phase column and fluorescence detection. Toxic effects were assessed by incorporation of [^{3}H]L-leucine into cellular proteins in the presence of capsazepine, the VR1 vanilloid receptor antagonist and Ruthenium red or tyrosine or calcium. Capsazepine (1 to 256 μM) did not modify the uptake rate of capsaicin for incubation times up to 24 h and did not antagonize capsaicin-induced protein synthesis inhibition. It rather inhibited protein synthesis per se from 100 to 256 μM. Ruthenium red which blocks mitochondrial calcium uptake, inhibited protein synthesis and did not antagonise or increase synergistically the effects of capsaicin. Interestingly in a medium deprived of calcium and supplemented by calcium chloride (10–50 μM) the protein synthesis inhibition induced by capsaicin is antagonised somehow. There was no prevention of capsaicin diffusion into the cells. Tyrosine, which seems to be the best preventive agent of capsaicin inhibitory effects, prevents its metabolism but not its diffusion. Capsaicin might enter cells by diffusion and interfere with protein synthesis machinery by competition with tyrosine which in turn prevents the metabolism of capsaicin. The results of the present study suggest that cell responses to capsaicin may be transduced through at least two molecular pathways, one involving VR1, since the receptor antagonist capsazepine fails to prevent the inhibitory effect of capsaicin in Vero cells of renal origin.

Key words Capsaicin · Capsazepine · Ruthenium red · Cytotoxicity · Vero cells

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Introduction

Capsaicin is a neurotoxin present in chillies and red peppers which are widely used as spices. Capsaicin is currently utilised to treat several pain disorders by topic application with a consequent transdermal passage (Kasting et al. 1997). The acute toxicity of capsaicin has been determined and showed, for example, in the mouse a large variation according to the route of administration: 0.56 (i.v.) to 190 (p.o.; mg/kg body wt.). However the mechanism of the lethal effects of capsaicin has not been elucidated. Recent studies have shown that hepatic cytochrome P450 2E1 (CYP 2E1) catalyses the conversion of capsaicin to reactive species such as the phenoxy radical intermediate capable of covalently binding to the active site of the enzyme as well as to cellular macromolecules (Gannett et al. 1990).

Using an expression cloning strategy, Caterina et al. (1997) isolated a functional cDNA encoding a capsaicin receptor from sensory neurons. This receptor (VR1, vanilloid receptor 1) is a non-selective cation channel structurally related to members of the Transient Receptor Potential (TRP) family of ion channels. The cloned capsaicin receptor is activated by increases in temperature in the noxious range, suggesting that it

functions also as a transducer of thermal stimuli in vivo. This could explain the way capsaicin enters cells before its numerous intracellular effects and metabolism. These authors also attested that capsaicin kills cells that express VR1. Within several hours of continuous exposure to capsaicin, HEK293 cells transfected with VR1 died, as determined morphologically and by staining with vital dyes. Furthermore, Caterina et al. (1997) have tested kidney cells and found by Northern blotting a mRNA species smaller than the one which has been found in the trigeminal and dorsal root sensory ganglia. Hence the difficulty to know if this transcript could encode a functional VR1 protein.

Our previous results have shown that capsaicin kills Vero cells with a 50% inhibitory concentration (IC_{50}) of 97 μM (Cochereau et al. 1997). This cytotoxicity (decrease of the number of viable cells in culture (Cochereau et al. 1997)) is mediated by inhibition of protein synthesis by competition with tyrosine, which in turn reverses this inhibition at a molar ratio of 4:1 (Cochereau et al. 1996). Literature data have shown that the effects of capsaicin can be antagonised by capsazepine (Dray et al. 1991; Urban and Dray 1991; Bevan et al. 1992) and Ruthenium red (Dray et al. 1990; Maggi et al. 1988). Taking into account all of the data on capsaicin cytotoxicity and the newly cloned capsaicin channel-receptor, several questions may be raised.

1. How does capsaicin enter cells?
2. Do Ruthenium red (RR) and capsazepine (a specific antagonist of capsaicin to VR1 receptor), prevent entry and/or metabolism and cytotoxicity?
3. What really kills cells and how is calcium involved?

The present experiments were designed to answer some of these questions. Vero cells from monkey kidney were chosen to confirm previous data and to compare with HEK 293 cells (of renal origin) transfected with the VR1 cDNA by Caterina et al. (1997).

Materials and methods

Chemicals

Capsaicin (8-methyl-N-vanillyl-6-nonenamide), Ruthenium red (Ruthenium oxychloride ammoniated; $Cl_6H_{42}N_{14}O_2Ru_3$) and tyrosine were obtained from Sigma (St. Quentin Fallavier, France). Capsaicin or tyrosine was dissolved in a 50:50 solution of ethanol and sterile 0.9% aq. sodium chloride. Ruthenium red was dissolved in ultra-pure water (MilliQ Plus; Millipore) made alkaline (pH 10.0) by 0.1 M NaOH. Capsazepine {2-[2-(4-chlorophenyl) ethylamino-thiocarbonyl]-7,8-dihydroxy-2,3,4,5-tetrahydro-1*H*-2-benzazepine} was obtained from RBI (Illkirch, France) and was dissolved in methanol.

Tyrosine and /or capsaicin was added to cell culture to reach the final concentrations of 1 to 776 μM and/or 10–250 μM respectively. The total volume of ethanol plus methanol never exceeded 1% of the total volume of culture. Effects were tested per se on protein synthesis at the final concentrations obtained in the culture medium. Both were found to be ineffective. Calcium chloride of the purest grade, obtained from Merck (France), was dissolved in ultra-pure water MilliQ Plus and added to culture medium at a final concentration of 10 to 50 μM in a volume not exceeding 0.5% of the culture medium volume. Chemical structures of capsaicin, tyrosine, capsazepine and Ruthenium red are shown in Fig. 1A–D.

Cell culture

Vero cells, from green monkey kidney (gift of BioMerieux, France) at the passage XXX, were routinely incubated in a humidified 95:5 air/CO_2 (%) mixture at 37 °C. Cells were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum, 4% L-glutamine (200 mM) and 100 μg/ml gentamicin. To study the influence of calcium, Dulbecco's modified Eagle medium (DMEM, D9785; Sigma) deprived of calcium was used, supplemented as indicated above in addition to given concentrations of $CaCl_2$, which were added to the medium prior to the cell culture.

Protein synthesis assay in Vero cells

Aliquots of cell-suspension (3 ml) were distributed in microplate wells (Greiner Labortechnick, Germany). Protein synthesis was assessed by the ability of cells to incorporate [^{3}H]L-leucine. The cells were cultured at a density of 4.0×10^5 per well and incubated 24 h later with capsaicin or capsaicin and tyrosine/Ruthenium red/capsazepine or calcium chloride for a further 24 h (incubation at 37 °C). [^{3}H]L-Leucine (1 μCi/ml) was added in each well for a 2-h pulse. Once the pulse was finished, the medium was removed, and cells were washed and harvested by scraping with a Falcon scraper. After centrifugation at 1000 *g* the cells were resuspended and sonicated for 20 s with a Bioblock sonicator at 60 mV in 300 μl of NaOH (20%) and all the samples were incubated for 20 min at 37 °C. Twenty microlitres of each homogenate was taken for total protein quantification using the method of Bradford (1976). The samples were maintained for 1 h at 4 °C after addition of 1 ml of trichloro-acetic acid (TCA, 40%). Lastly the samples were filtered on 3MM Whatman microfibre filter discs, which were dried at 70 °C for 20 min. The radioactivity on the paper filters was counted with a liquid scintillation analyser, a Packard 2000-CA spectrophotometer after addition of 5 ml of Beckman Ready Organic supplemented by 0.9% acetic acid. The cpm values were adjusted with the total protein content in each sample quantified using Bradford's technique (1976).

Three series of controls were performed, one without any of the following: capsaicin or tyrosine and capsaicin or capsazepine or Ruthenium red, the second with only capsaicin at IC_{50} (97 μM) and the third with large amounts of tyrosine (388–776 μM), capsazepine, (100–256 μM) and Ruthenium red (10–500 μM). Calcium was added when needed at 10 to 50 μM. The counts were normalized to total protein (mg).

Fluorescence imaging

Cells were loaded with the calcium indicator Fura-2 via incubation with its acetoxymethyl ester (5 μM, 20 min at 37 °C), washed and incubated with capsaicin in the presence of different concentrations of calcium. Observations were made at given times up to 24 h using a fluorescent microscope [Hund 500, Germany; fluorescein isothiocyanate (FITC), tetramethylrhodamine B isothiocyanate (TRITC)] coupled to a JVC colour video camera, and image analysis software to capture the fluorescent image for further analysis.

Extraction and HPLC-determination of capsaicin and tyrosine

Extraction of capsaicin and tyrosine from ethanol lysed cells was carried out as follows: 1 ml of absolute ethanol was added to cell pellets (10 min centrifugation at 4000 *g*) and were sonicated twice for 20 s at 60 mV. After 2 h at + 8 °C, all samples were centrifuged again under the same conditions for 10 min The supernatants (40 μl) were chromatographed by HPLC in the following conditions after filtration on 0.22 μm pore size filters from Millipore-

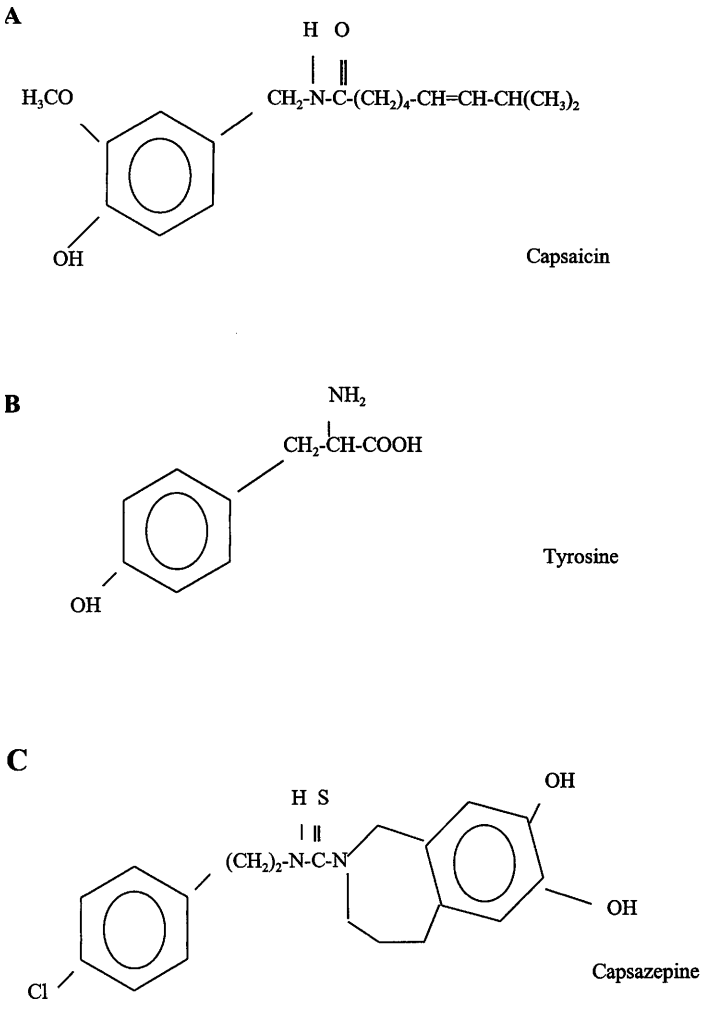

Fig. 1 Structure of **A** capsaicin, **B** tyrosine, **C** capsazepine, and **D** Ruthenium red

Waters:C18-Pronctosil (5 μm) column and acetonitrile:water (60:40, v/v) as eluant. Flow rate was 1.15 ml/min.

HPLC-determination of capsaicin was carried out by recording the fluorescence at 330 nm after excitation at 270 nm (Saria et al. 1981) followed by integration using computer-aided software, PIC3, developed by ICS (Instrumental Consumable Service) according to the areas under the curve; capsaicin standard solutions were assayed sequentially. Capsaicin was also quantified in the culture medium, soon after addition and after 24 h incubation by loading directly 40 μl of medium on the HPLC column through a pre-column containing C18-Spherisorb ODS_2. The intracellular volume is taken as the difference between the total volume (cells + medium) and the culture medium volume (the volume of cells-pellets after centrifugation at 4000 *g*, 5 min at 4 °C) even if this also contains organelles and membranes.

HPLC conditions for tyrosine quantification were: C18-Spherisorb ODS_2 column and pre-column, phosphate buffer 20 mM, (pH 6.8):methanol (85:15, v/v) as eluant A ; methanol:water (70:30, v/v) as eluant B ; flow rate 1 ml/min. A gradient was run from 100% of A to 100% of B within 50 min The HPLC-determination was carried out by reading the absorbance at 340 nm of the *o*-phthalaldehyde-β-mercaptoethanol (OPA-βME)-derivated amino-acid according to the method developed by (Joseph and Marsden, 1986). The same HPLC-PIC3 processing was used for quantification of selected amino-acid-peaks according to surfaces under curve, and tyrosine standard solutions assayed sequentially.

Statistical analysis

The results were expressed as mean ± SEM before a statistical analysis performed using the non-parametric test of Mann-Whitney (Gad and Weil 1982).

Results

In agreement with our previous studies (Cochereau et al. 1997), capsaicin inhibited protein synthesis in Vero cells.

The inhibition was reversed by tyrosine when the molar ratio of 4/1 (tyrosine versus capsaicin) was reached (Fig. 2). Capsazepine, the capsaicin antagonist to VR1, was able per se to inhibit protein synthesis at a concentration of 100 μM in the medium (Fig 3). When capsazepine was added to the culture medium (1 to 256 μM) in the presence of capsaicin (97 μM, corresponding to the IC_{50}) no preventive effect of capsazepine against capsaicin inhibition of protein synthesis was observed (Fig. 3).

In order to study the influence of calcium on capsaicin-induced inhibition of protein synthesis, cells were incubated with Ruthenium red alone and in the presence of capsaicin in the medium. Ruthenium red, a very effective and relatively specific blocker of mitochondrial calcium uptake, was able per se (at a concentration of 100 μM) to inhibit protein synthesis, which was almost suppressed when cells were incubated with a concentration of 500 μM (Fig. 4). Thus, this compound was found to be almost as inhibitory as capsaicin. When added to the medium in the presence of capsaicin at concentrations per se ineffective, Ruthenium red failed to antagonise capsaicin inhibitory effects and no synergism was observed between the two compounds (Fig. 4). This suggests that mitochondrial calcium uptake is not involved, at least directly, in capsaicin-mediated toxic effects in this cell-line.

Protein synthesis was also studied by incorporating tritiated leucine into the proteins of Vero cells in the presence of increasing concentrations of $CaCl_2$ (10 to 50 μM). This study showed that calcium, added to the culture medium slightly but significantly inhibited protein synthesis from the concentration of 20 μM (Table 1). $CaCl_2$ (50 μM) alone already significantly inhibited protein synthesis by ~20% compared to the control without capsaicin or without calcium.

In another series of experiments $CaCl_2$ was added (10 or 50 μM) to low and high concentrations of capsaicin in the culture medium of Vero cells. $CaCl_2$ did not show a clear protective effect on the capsaicin-induced inhibition of protein synthesis except at high concentrations of the toxin ($P < 0.001$). With 250 μM capsaicin, $CaCl_2$ (50 μM) showed a protection (Fig. 5). This protective effect was also observed with 125 μM capsaicin.

Thus it was decided to further study the influence of calcium on the capsaicin extracellular and intracellular concentrations and protein synthesis inhibition with

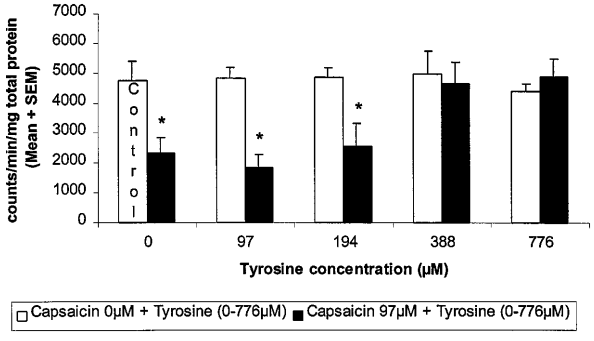

Fig. 2 Effects of tyrosine against the capsaicin-induced inhibition of protein synthesis in Vero cells. Vero cells, distributed 24 h previously at 4.0×10^5 cells/ml in 3-ml wells, were incubated for 24 h with 97 μM capsaicin (except for the control) and with different concentrations of tyrosine (97, 194, 388, 776 μM). Protein synthesis was assessed by incorporation of [^{3}H]L-leucine during a 2-h pulse. The counts/min were normalized to total protein (mg). * Significantly different from control, $P < 0.001$

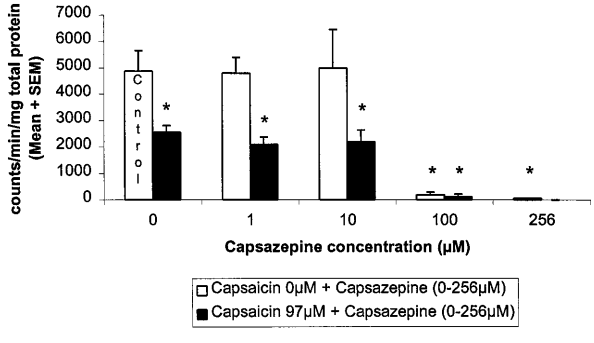

Fig. 3 Protein synthesis in Vero cells followed by incorporation of [^{3}H]L-leucine during a 2-h pulse after 24 h incubation with capsazepine (1–256 μM). Vero cells, distributed 24 h previously at 4.0×10^5 cells/ml in 3-ml wells, were incubated for 24 h with 97 μM capsaicin (except for the control) and with different concentrations of capsazepine (1–256 μM). Further details are as given in the legend to Fig. 2. * Significantly different from the control, $P < 0.001$

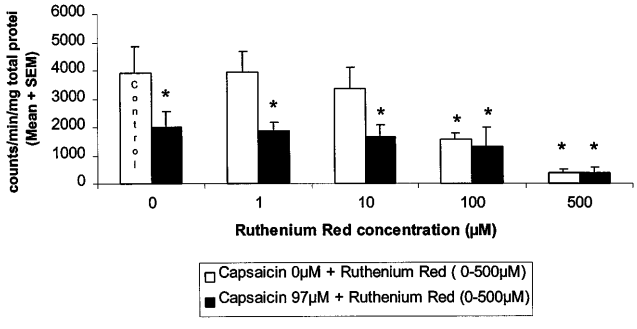

Fig. 4 Protein synthesis in Vero cells followed by incorporation of [^{3}H]L-leucine during a 2-h pulse after 24 h incubation with Ruthenium red (1–500 μM). Vero cells, distributed 24 h previously at 4.0×10^5 cells/ml in 3-ml wells, were incubated for 24 h with 97 μM capsaicin (except for the control) and with different concentrations of ruthenium red (1–500 μM). Further details are as given in the legend to Fig. 2. * Significantly different from the control, $P < 0.001$

Table 1 Protein synthesis in Vero cells incubated in Dulbecco's modified Eagle medium (DMEM, D9785) supplemented with increasing concentration of $CaCl_2$ (10 to 50 μM). The incorporated radioactivity (cpm) was calculated as a mean of two serial experiments run in triplicate ($n = 6$) as cpm ± SEM and the counts/min were normalised to total protein (mg)

Control	$CaCl_2$ (μM)			
0	10	20	40	50
4166 ± 688	3274 ± 456	2804 ± 320	2632 ± 470	2389 ± 394

only 10 and 50 μM of calcium. The intracellular concentration of capsaicin was determined in our experimental conditions together with its concentration in the culture medium in order to establish the balance and to correlate with the inhibitory effects. After 24 h incubation of Vero cells with capsaicin alone, ~50% of the toxin added was still present in the medium as the native form whereas 20–25% was inside the cells also as the native form (Table 2).

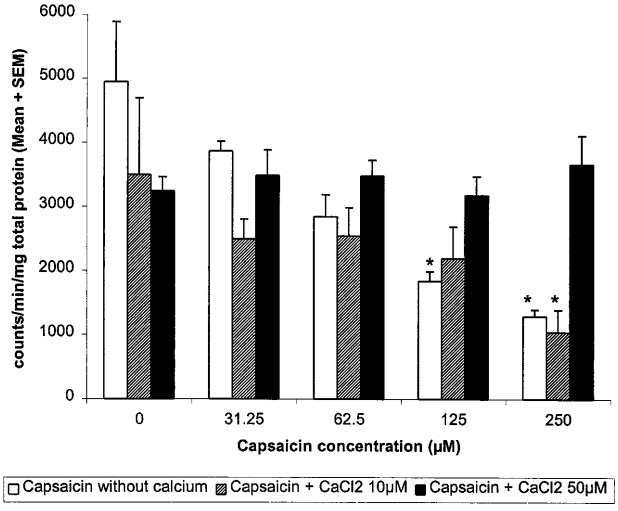

Fig. 5 Effects of $CaCl_2$ (10, 50 μM) on the inhibition of protein synthesis induced by capsaicin in Vero cells followed by incorporation of [^{3}H]L-leucine. The incorporated radioactivity (cpm) was calculated as a mean of two serial experiments run in triplicate ($n = 6$) as cpm ± SEM. The counts/min were normalized to total protein (mg). * Significantly different from the corresponding capsaicin-alone culture ($P < 0.001$)

Very likely 25 to 30% of the added capsaicin was transformed into a completely different compound, which was not detectable under the same HPLC conditions (Fig. 6). When tyrosine was added to the culture medium in the presence of capsaicin, the concentration in the medium reflected the quantity added (Table 2). The intracellular concentrations rather reflected the ratio (tyrosine/capsaicin); when the ratio was >4/1, the intracellular concentration was 11.5% of the loaded concentration; when this ratio was equal to 4/1, 50% of the capsaicin was in the cells and when it was <4/1 (for example 388 μM tyrosine and 194 μM capsaicin), the intracellular concentration decreased.

The balance (internal + external concentration) was almost 100% of the quantity added indicating that the toxin is probably not metabolized in the presence of tyrosine (Table 2). In the absence of tyrosine capsaicin was partially metabolized. In the presence of capsazepine, a negative balance was found for capsaicin whatever the concentration of the antagonist. However with 100 μM of capsazepine the concentration of capsaicin was higher in the medium and lower in the cells, with a more negative balance indicating the transformation of larger amounts of the toxin (Fig . 7A). With Ruthenium

Table 2 Concentration of capsaicin (μM) in the culture medium and in the intracellular compartment as determined by high-performance liquid chromatography (HPLC) separation and quantification. Cells were incubated with 1/2 to twice the IC_{50} concentration of capsaicin in the presence of 97 to 388 μM tyrosine

Capsaicin and/or tyrosine added to culture medium (μM)	0	Tyrosine, 388 μM	Capsaicin, 97 μM	Capsaicin, 48.5 μM + tyrosine, 388 μM	Capsaicin, 97 μM + tyrosine, 388 μM	Capsaicin, 194 μM + tyrosine, 388 μM
Capsaicin in culture medium after 24 h (μM)	0	0	43.8 ± 2.9	43.2 ± 3.7	48 ± 8.2	152.7 ± 6.3
Intracellular capsaicin after 24 h (μM)	0	0	22.8 ± 4.2	5.6 ± 1.8	48 ± 5.9	37.5 ± 15.8

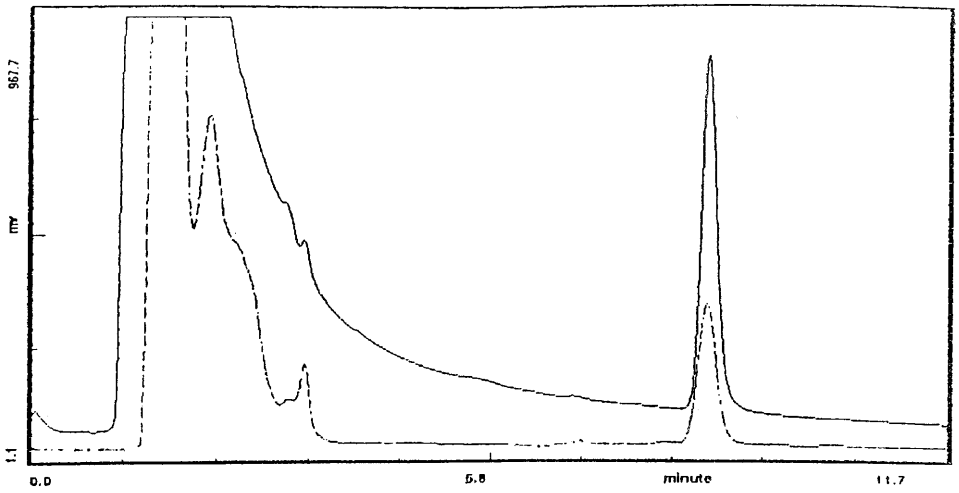

Fig. 6 High-performance liquid chromatography (HPLC) elution profile of capsaicin in Vero cells treated with 97 μM capsaicin + 388 μM tyrosine. Analysis of capsaicin was carried out as described in the Materials and methods. *Solid line*, Capsaicin in culture medium; *dashed line*, capsaicin in cells

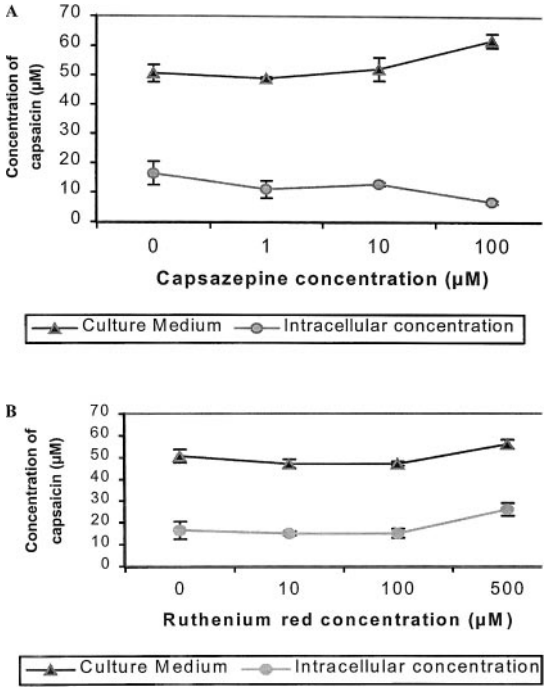

Fig. 7 Concentration of capsaicin in the culture medium and in the intracellular compartment as determined by HPLC separation and quantification. Cells were incubated with the IC_{50} concentration of capsaicin (97 μM) in the presence of : **A** capsazepine (1 to 100 μM), **B** Ruthenium red (10–500 μM)

red, the balance of capsaicin was negative in all cases except that the intracellular concentration of capsaicin was fourfold higher with 500 μM of RR compared to 100 μM of capsazepine indicating a smaller rate of metabolism (Fig. 7B).

The determination of capsaicin concentration in the culture medium after 24 h of incubation with increasing concentrations of calcium chloride (10–50 μM) has revealed that for the IC_{50} concentration of capsaicin (97 μM) added, the detected quantities in the culture medium were respectively 43.5 ± 3, 47.5 ± 2, 52.5 ± 0.5, 60.5 ± 2 μM when 10, 20, 40 and 50 μM of calcium chloride was present. On the other hand, the intracellular capsaicin concentration did not vary in the same way and was found to be respectively 26.5 ± 0.5, 23.8 ± 3, 21 ± 3 and 18 ± 4.5 when 10, 20, 40 and 50 μM of calcium chloride were present. Intracellular calcium evaluated by fluorescence due to Fura-2 was not modified when capsaicin and calcium were present simultaneously (results not shown).

Discussion

The results of the present study show that capsaicin inhibits protein synthesis, and that the inhibition can be prevented by tyrosine in Vero cells, thus confirming our previous results (Cochereau et al. 1996; 1997). Concerning the uptake of capsaicin by the cells, the results indicate that capsaicin might cross the plasma membrane mainly by diffusion. No binding to a specific receptor occurred since capsazepine failed to prevent the entry of the toxin, its metabolism and protein synthesis inhibition. Instead, tyrosine prevented the inhibition of protein synthesis induced by capsaicin in Vero cells, but there was no competition for entry between the two compounds. The competition was only inside the cells for metabolism and protein synthesis. For capsaicin the uptake seemed not to be saturable (Table 2) even in the presence of tyrosine.

Concerning the protein synthesis inhibition it is clearly understood that capsaicin prevents the aminoacylation of tyrosine (Cochereau et al. 1996) and hence the elongation step of protein synthesis machinery. It is to be noted that the uptake of leucine was not impaired by capsaicin (result not shown). The same finding applies for tyrosine which once inside the cells, prevented the capsaicin-induced inhibition of protein synthesis.

Concerning the metabolism of capsaicin, the decrease of metabolism occurred at the same time as the decrease of inhibitory effects suggesting that the metabolites are cytotoxic. This is a new aspect of the cytotoxicity of capsaicin which needs further investigation. However the fact that mainly metabolites are responsible for the inhibitory effects is confirmed by experiments performed in the presence of capsaicin plus capsazepine and capsaicin plus Ruthenium red. In these experiments, run separately, metabolism of the toxin was observed together with a lack of protective effects against the inhibition of protein synthesis induced by capsaicin. Our data showed clearly that capsaicin was metabolized inside the cells, since the balance of intracellular and extracellular capsaicin was always negative. The missing capsaicin was likely metabolized into reactive form(s) bound to cellular components as previously suggested by Surh and Lee (1995) or transformed into non-extractable metabolites. This suggestion is supported by the results in the literature (Gannett et al.1990; Dray et al. 1990; Holzer 1991); however, it appeared that the rate of metabolism varied according to the presence of compounds, such as calcium, tyrosine, capsazepine, and Ruthenium red.

The data from the literature have shown the existence of a specific receptor (VR1) involved in capsaicin-mediated effects (Caterina et al. 1997; Dray et al. 1991; Urban and Dray 1991; Bevan et al.; 1992; Holzer 1991; Szallasi 1994). In particular, capsaicin-evoked responses in VR1-expressing oocytes are reversibly blocked by the competitive vanilloid receptor antagonist capsazepine at IC_{50} of 283.5 nM (Caterina et al. 1997). Another pharmacological characteristic of vanilloid receptors is their sensitivity to the non-competitive antagonist Ruthenium red, which blocks capsaicin-evoked responses in a reversible manner (Dray et al. 1990; Maggi et al. 1988). Our findings show that the effects of capsaicin in Vero cells are not prevented by capsazepine, a specific antagonist of VR1 receptor, thus indicating that the toxic effect of this compound is not mediated by interaction

with a specific vanilloid receptor. Cell death occurring via this receptor has been reported by (Caterina et al. 1997) in HEK293 cells. The authors reasoned that a capsaicin receptor-encoding cDNA might confer to non-neuronal cells the ability to undergo increases in intracellular free calcium upon exposure to capsaicin; this supposition assumes that capsaicin acts at a proteinaceous site and that a single cDNA can confer sensitivity to capsaicin in a heterologous context.

The apparent discrepancy between our results and those of Caterina et al. (1997) can be explained by the lack of capsaicin receptors in Vero cells and/or by a different pathway involved in the toxic effect in our cell system. We have shown that the capsaicin toxic effect is strictly related to inhibition of protein synthesis and does occur through a pathway which, apparently, does not involve (at least directly) mitochondrial Ca^{2+} buffering action or calcium movement through the plasma membrane. In fact Ruthenium red, a stain which is a very effective and relatively specific blocker of mitochondrial Ca^{2+} uptake (Miller 1991), does not interfere with capsaicin effects, i.e. RR does not prevent the inhibition of protein synthesis induced by the toxin. RR becomes cytotoxic per se at high concentrations and, at low concentrations, does not synergize with capsaicin. However, it should be pointed out that, despite a large capacity to accumulate Ca^{2+}, mitochondria do not capture much Ca^{2+} when $[Ca^{2+}]i$ is maintained within the normal dynamic physiological range ($\sim$0.1–1 μM), the reason being that their affinity for Ca^{2+} and the rate of Ca^{2+} uptake are very low under normal physiological conditions (Llinas and Moreno 1998). Only under pathological conditions of Ca^{2+} overload (when $[Ca^{2+}]i$ exceeds 5 μM), will mitochondria capture substantial amounts of Ca^{2+}. This does not happen with capsaicin, since video imaging with Fura-2 did not reveal any increase in intracellular fluorescence nor compartmentalization in the presence of this toxin.

Calcium per se, when exceeding 5 μM (i.e 10 μM) was inhibitory to protein synthesis. Interestingly, calcium protected cells from capsaicin-induced inhibition of protein synthesis at concentrations which were not per se inhibitory. These results could be explained, at least partially, by assuming that the activity of Ca^{2+}-dependent enzymes, possibly affected by high concentrations of capsaicin, can be restored. This would imply that calcium could prevent diffusion of capsaicin into cells. This is not the case under our experimental conditions; we therefore may hypothesize the existence in the endoplasmic reticulum of a low-affinity binding site of Ca^{2+} on a proteinaceous entity also binding capsaicin or tyrosine similarly to that on aminoacyl-tRNA synthetases, which also bind Mg^{2+} and the amino acid (Cochereau et al. 1996). Another possible explanation, assuming binding of capsaicin with calcium (or complex formation), is that capsaicin itself could behave as a buffering system such that no calcium would be available for the activity of Ca^{2+}-dependent enzymes implicated in the production of energy needed for protein synthesis. Such a mechanism could explain why calcium overload in the presence of capsaicin would counteract the inhibition of protein synthesis, thus protecting cells. Another argument which could be put forward is supported by Chard et al. (1995) who found that calcium-activated proteases are involved in the neurotoxicity of capsaicin in neurons of the dorsal root ganglion. This could account, at least partially, for the inhibition of protein synthesis and cytotoxicity in the presence of Ca^{2+} and capsaicin.

The fact that capsaicin can interfere with enzyme activity is not surprising since this compound has been shown to inhibit NADH oxidase (Morré et al. 1995) and protein synthesis (Cochereau et al. 1997) in cell cultures. Collectively, our results show that only tyrosine and, though to a lesser extent, calcium are able to prevent the protein synthesis inhibition induced by capsaicin, whereas capsazepine and RR failed. In our cell culture system the main mechanism of the cell damage by capsaicin still remains inhibition of protein synthesis and the most effective prevention occurs with the amino acid tyrosine. Focusing on these aspects is of major importance since capsaicin is currently used to treat several pain disorders by topical application. Transdermal passage from topically applied preparations dosed at 0.025 and 0.075% is likely in the range of 3 to 5%, 24 h after a single application (Kasting et al. 1997; Jolleys 1989; Marks 1989). Since capsaicin preparations could be applied several times per day, substantial amounts of the compound are likely to enter the body which can adversely affect all kinds of tissues and not only cells from the central or peripheral nervous system.

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