RESEARCH ARTICLE

Neuroprotection induced by vitamin E against oxidative stress in hippocampal neurons: Involvement of TRPV1 channels

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Pretreatment of cultured hippocampal neurons with a low concentration of alpha-tocopherol (α-TP), the major component of vitamin E, results in a long-lasting protection against oxidative damages, *via* genomic effects. This neuroprotection is associated with the attenuation of a calcium influx triggered by oxidative agents such as Fe^{2+} ions. This Ca^{2+} influx is supported by a TRP-like channel, also partly involved in capacitive calcium entry within neurons. Here, we evidence the contribution of TRPV1 channels in this mechanism. TRPV1 channels are activated by various agents including capsaicin, the pungent component of hot chili peppers and blocked by capsazepine (CPZ) or 5′-iodo-resiniferatoxin. Both TRPV1 inhibitors strongly reduced Fe^{2+} ion-mediated toxicity and Ca^{2+} influx, in the same way as to α-TP pretreatment. Moreover, CPZ also decreased capacitive calcium entry in hippocampal neurons. Finally, both CPZ and 5′-iodo-resiniferatoxin reduced spontaneous excitatory synaptic transmission; this depression of synaptic transmission being largely occluded in α-TP-pretreated neurons. In conclusion, in our experimental model, TRPV1 channels are involved in the Fe^{2+} ion-induced neuronal death and a negative modulation of this channel activity by α-TP pretreatment may account, at least in part, for the long-lasting neuroprotection against oxidative stress.

Keywords:
α-Tocopherol / Hippocampal neurons / Neuroprotection / Oxidative stress / TRPV1

1 Introduction

Vitamin E is an essential compound in human nutrition, required for proper cellular functioning, especially through its antioxidant activity [1]. Indeed, in nervous cells, vitamin E is the major scavenger of radical-oxygenated species along to reduced glutathione [2]. Consequently, deficits in Vitamin E lead to improper brain development and function. In fact, brain aging and neurodegenerative disorders such as Alzheimer disease are generally associated with an increase in oxidative stress markers and a decrease in vitamin E levels [3, 4]. Therefore, restorations of vitamin E levels consist in interesting protective strategies to prevent or delay the occurrence of neurodegeneration involving oxidative stress. However, up to date, clinical studies based on dietary supplementation with vitamin E in Alzheimer patients lead

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Abbreviations: α-TP, alpha-tocopherol; **CCE,** capacitive calcium entry; **CPZ,** capsazepine; ***Ih* current,** hyperpolarization-activated cationic current; **I-RTX,** 5′-iodo-resiniferatoxin; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; **pre-α-TP cells,** α-TP pretreated cells; **sEPSC,** spontaneous excitatory postsynaptic current; **TRP,** transient receptor potential channel; **TRPV1,** vanilloid 1 TRP channel subtype; **ZD7288,** 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium choride

to controversial conclusions regarding beneficial effects of such supplementation, especially in delaying the onset of severe pathological manifestations [5]. These controversies may result from an improper use of vitamin E in human studies, since molecular mechanisms underlying vitamin E protective actions are not thoroughly deciphered [6]. In fact, natural vitamin E is a mixture of eight different compounds: α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol [7–9]. Among them, α-tocopherol (α-TP) is the most abundant one and thus exhibits the highest biological activity [1]. In addition, its bioavailability is higher than that of its congeners because of the presence of a specific α-TP transport protein, which facilitates its delivery to liver and plasma [10]. Beneficial effects of α-TP do not only rely on its antiradical activity but also involve other molecular mechanisms, including some genomic actions [11]. In this respect, we recently demonstrated on cultured hippocampal neurons that an application of a low concentration of α-TP results in an enduring protection against oxidative insults triggered by exposure to Fe^{2+} ions or hydroperoxides, this protection being lost under the blockade of protein synthesis by cycloheximide [12]. Other reports indicate that α-TP is actually able to stimulate *de novo* protein synthesis in various cell types [13–15]. We found that the α-TP pretreatment of hippocampal neurons impairs the intracellular Ca^{2+} increase elicited by an oxidative insult. Indeed, while Fe^{2+} ions trigger an intracellular Ca^{2+} overload leading to cell death, they only moderately enhance intracellular Ca^{2+} in cells previously treated with low concentration of α-TP [16]. Further pharmacological investigations indicate that this Ca^{2+} influx elicited by oxidative stress occurs mainly through nonselective cationic channels belonging to the transient receptor potential channel (TRP) family in hippocampal neurons [16], consistent with findings in other experimental models [17]. Indeed, the blockade of these channels with nonspecific blockers, such as Ruthenium Red, Gd^{3+}, and La^{3+} ions, inhibits both Fe^{2+} ion-induced Ca^{2+} overload and neurotoxicity. Here, we attempt to go deeply into the characterization of the TRP subtype involved in this mechanism. We examined the contribution of the vanilloid 1 TRP subtype channel (TRPV1) to the neurotoxicity of Fe^{2+} and its possible modulation by the α-TP pretreatment. Indeed, in our hands, the exposure of cells to the TRPV agonist, 2-aminoethoxydiphenylborane results in cell death and potentiates the increase in the Fe^{2+} ion-elicited calcium [16]. In addition, activation of these channels with the selective agonist capsaicin also triggers the death of mesencephalic neurons both *in vitro* and *in vivo* [18–20].

2 Materials and methods

All experiments were carried out in accordance with the European Communities Council Directive of 24th November 1986 (86/609/ECC). Sprague–Dawley rats were from the Centre d'Elevage Depré (France). Versene, antibiotics, and fetal calf serum were purchased from Invitrogen. Culture media (DMEM and Ham F12) and Dulbecco-modified phosphate-buffered saline (Dulbecco's PBS) were from Eurobio. Culture dishes were from Nunc. Capsazepine (CPZ), capsaicin, and ZD 7288 (4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride) were purchased from Tocris Cookson. NBQX and D-AP5 were from Ascent. 5′-Iodo-resiniferatoxin (I-RTX) was from LC Labs. All other chemicals were from Sigma.

2.1 Hippocampal neuron-enriched cultures

Primary neuronal cultures were established from 18-day-old embryonic rat hippocampi, as previously described [21, 22], with minor modifications. After preincubation with Versene, hippocampal cells were mechanically dissociated and plated at a density of 5×10^5 cells/dish in 24-well dishes for cell survival measurements. Alternatively, cells were plated at a density of 2×10^6 cells/dish in 8-well dishes containing either ThermanoxTM coverslips (Nunc) for electrophysiology experiments or glass coverslips for intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement. All culture dishes have been previously coated with poly-L-lysine (75 μg/mL) and then with DMEM/HAM F12 containing 10% fetal calf serum. Cells were grown in a defined medium containing DMEM/HAM F12 supplemented with 33 mM glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 mM HEPES, 13 mM sodium bicarbonate, 50 μg/mL transferrin, 87 μM insulin, 1 pM β-estradiol, 3 nM triiodothyronine, 20 nM progesterone, 46 nM sodium selenite, and 100 μM putrescine. All experiments were performed on cell cultures grown for at least 2 wk *in vitro*.

2.2 Cell culture treatments

Solutions of $FeSO_4$ and α-TP were prepared extemporaneously for each experiment. Whenever possible, treatments were performed using aqueous concentrated solutions (usually 100 times) directly added to the culture medium. When necessary, concentrated (usually 1000 times) solutions of the drugs were prepared in DMSO. Control experiments have been performed with DMSO. At concentrations up to 0.5%, DMSO did not significantly affect cell survival in hippocampal cultures (data not shown). Pretreatments with α-TP were performed by applying this drug for 72 h. To induce oxidative stress, $FeSO_4$ was applied for 24 h for cell viability assays or 30 min for $[Ca^{2+}]_i$ measurements.

2.3 Cell viability assay

Cell viability was determined by measuring mitochondrial activity using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), as previously described [23].

Briefly, cells were washed with 500 μL of Krebs-Ringer buffer comprising 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-glucose, and 10 mM HEPES (bubbled with O_2/CO_2: 95/5), and then MTT (250 μg/mL) was added. After incubation at 37°C for 20 min, the incubation medium was discarded and cells were lysed with DMSO. Optical density (OD) at 570 nm was measured and data were expressed as the percentages of control OD obtained with untreated cells. The statistical significances of the differences between experimental and control data were assessed with SigmaStat Software (Jandel) using two-way ANOVA, followed by Holm-Sidak's *post hoc* test ($p < 0.05$ considered significant and indicated by *).

2.4 Measurements of cytosolic-free Ca^{2+} concentration

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured with the fluorescent indicator fura-2 [16, 24, 25]. For this purpose, hippocampal cells grown on square (10 × 10 mm) glass coverslips were loaded with fura-2 by a 30 min-incubation at 37°C with 5 μM fura-2-AM and 0.02% Pluronic in a modified extracellular solution comprising 125 mM NaCl, 0.5 mM KCl, 0.3 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM D-glucose, 17 mM HEPES saturated with O_2/CO_2 (95/5). $[Ca^{2+}]_i$ was monitored by videomicroscopy. After rinsing, the glass coverslip was transferred to a recording chamber mounted on an inverted microscope (Leica, DMIRB). Fura-2 emission was obtained by alternatively exciting the preparation at 340 and 380 nm with a rotating filter wheel (Sutter Instruments) and by monitoring fluorescence emissions (F340 and F380, respectively) at 510 nm. The ratio of emissions at 510 nm (F340/F380) was recorded every 30 or 60 s. Fluorescent signals were collected with a CCD camera (Hamamatsu), digitized, and analyzed with image analysis software (Acquacosmos, Hamamatsu). The coverslips were continually superfused with the modified extracellular solution and thermostated at 37°C. The '*n*' value represents the entire population of cells recorded from at least three independent cultures. Since values of $[Ca^{2+}]_i$ were not normally distributed [16],] statistical analyses were performed using Kruskal–Wallis ANOVA on ranks, followed by Dunn's *post hoc* test ($p < 0.05$ considered significant and indicated by *).

2.5 Electrophysiology

On the day of the experiment, a coverslip was transferred to the recording chamber of an upright microscope (DMLFS, Leica). Cells were superfused (flow rate ~5 mL/min) with the extracellular solution containing 124 mM NaCl, 3 mM KCl, 26 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-Glucose, and 10 mM HEPES (bubbled with O_2/CO_2: 95/5). All experiments were carried out at room temperature. Drugs were directly applied in the perfusate. All signals from whole-cell recordings were measured and digitized with double patch-clamp amplifier (EPC9/2, HEKA, Germany).

To facilitate excitatory transmission, recording of excitatory spontaneous transmission (sEPSCs) was performed using an Mg^{2+}-free extracellular solution supplemented with 10 μM glycine. Spontaneous excitatory postsynaptic currents (sEPSCs) were measured using whole-cell recording by holding the voltage at −60 mV with glass microelectrodes (4–7 MΩ resistance) filled with an intracellular solution comprising 120 mM $CsMeSO_3$, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM *N*-(2,6-dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide (QX-314), 5 mM HEPES (pH = 7.3), and 4 mM Mg-ATP [26]. Digitized sEPSC signals were filtered at 1 kHz and sampled at 10 kHz and then analyzed with John Dempster's softwares 'WinEDR' (Strathclyde University).

To record hyperpolarization-activated cationic currents (*Ih* currents), microelectrodes were filled with an intracellular solution containing 130 mM Kgluconate, 1 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 5 mM HEPES (pH = 7.3), and 4 mM Mg-ATP. Hyperpolarizing steps of the holding potential from −60 to −120 mV (1 s duration) were produced every 30 s in order to generate *Ih* currents.

Data are presented as mean ± SEM on graphs plotting pooled data. Statistical significances of the differences between experimental and control data were assessed using two-way ANOVA, followed by Holm–Sidak's *post hoc* test ($p < 0.05$ considered significant and indicated by *).

3 Results

3.1 Neuroprotective action of TRPV1 antagonists against oxidative stress

The TRPV1 antagonists, CPZ, and I-RTX, were tested for their ability to protect hippocampal neurons against Fe^{2+} ion-mediated oxidative stress. Both CPZ (Fig. 1) and I-RTX (Fig. 2) induced a concentration-dependent prevention of neuronal death elicited by the exposure to Fe^{2+} ions. As already shown for the broad range TRP channel blockers, Gd^{3+} ions, La^{3+} ions, and ruthenium red, the effects of CPZ and I-RTX were mainly competitive against the Fe^{2+} ion toxicity. Complete protections were obtained by applying CPZ and I-RTX at 1 and 3 μM, respectively.

3.2 Effect of CPZ on the Fe^{2+}-elicited Ca^{2+} overload in hippocampal neurons

As previously shown [16], Fe^{2+} ions induced a time-dependent increase in $[Ca^{2+}]_i$ in cultured hippocampal neurons (Fig. 3). After a 30 min exposure to 50 μM $FeSO_4$, this

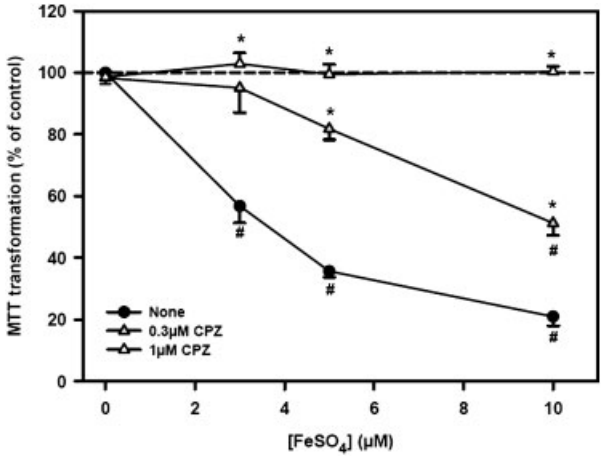


Figure 1. CPZ protects neurons against Fe^{2+} ion-mediated cellular injury. CPZ (0.3 and 1 μM) was co-applied together with different concentrations of $FeSO_4$ (ranging from 2 to 10 μM). Cell survival was measured 24 h after the $FeSO_4$ application using MTT assays as described in the Section 2. On the graph, data are mean ± SEM of at least three independent determinations each performed in triplicate. They are expressed as percentages of MTT transformation obtained in control culture without any treatment. $^*p<0.05$ *versus* $FeSO_4$ alone; $\sharp p<0.05$ *versus* respective control.

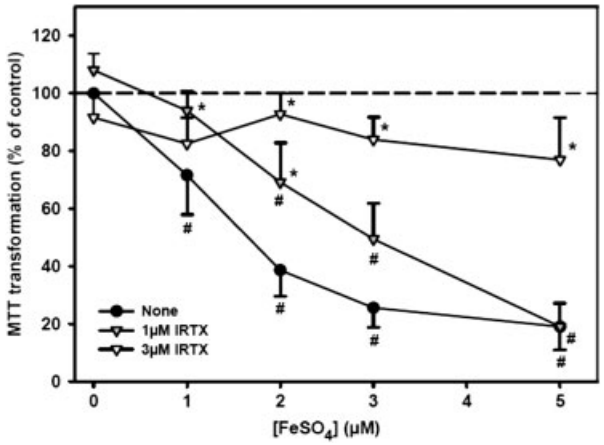
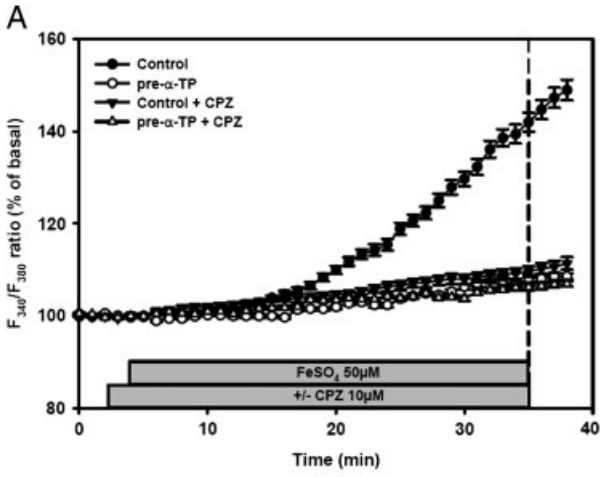


Figure 2. I-RTX protects neurons against Fe^{2+} ion-mediated cellular injury. I-RTX (1 and 3 μM) was co-applied together with increasing concentrations of $FeSO_4$. Cell survival was measured 24 h after the $FeSO_4$ application. On the graph, data are mean ± SEM of at least three independent determinations each performed in triplicate. They are expressed as percentages of MTT transformation obtained in control culture without any treatment. $^*p<0.05$ *versus* $FeSO_4$ alone; $\sharp p<0.05$ *versus* respective control.



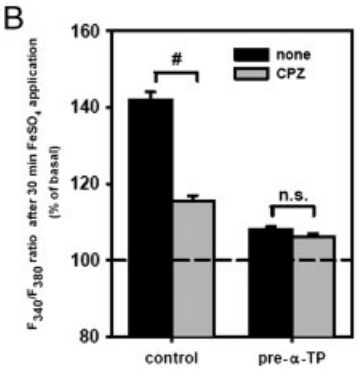


Figure 3. CPZ impairs the intracellular Ca^{2+} overload elicited by Fe^{2+} ions in hippocampal neurons. Intracellular Ca^{2+} concentration of hippocampal neurons either pretreated or not with α-TP (1 μM, 72 h) was monitored using fura-2 fluorescence imaging during an exposure to 50 μM $FeSO_4$ for at least 30 min, either in the presence or in the absence of 10 μM CPZ. Following fura-2 loading into neurons, as described in Section 2, the ratio of fura-2 fluorescence (F340/F380 ratio) was measured at 510 nm under alternating excitations at 340 and 380 nm. Earlier experiments indicated that α-TP pretreatment did not significantly modify the basal-free calcium concentration within neurons [16]; thus, F340/F380 ratios were normalized to basal F340/F380 ratio obtained by monitoring $[Ca^{2+}]_i$ for 3 min before any drug application. Data are expressed as mean ± SEM (A) Time courses of $[Ca^{2+}]_i$ increase in response to 50 μM $FeSO_4$ in control and pre-α-TP cells, either in the absence ($n = 280$ and 92, respectively) or in the presence of 10 μM CPZ ($n = 163$ and 89, respectively). (B) Recapitulative graph plotting $[Ca^{2+}]_i$ increases obtained following a 30 min-exposure to Fe^{2+} ions, according to the different treatments (time indicated by the dotted line in A). $\sharp$ $p<0.05$ *versus* the respective value in the absence of CPZ.

increase reached 42 ± 2% ($n = 280$) above basal in control neurons. However, in the presence of 10 μM CPZ, this enhancement of $[Ca^{2+}]_i$ was largely impaired since it reached only 16 ± 1% ($n = 163$) above basal (Fig. 2A). Furthermore, as shown earlier, in α-TP-pretreated (pre-α-TP) cells, $[Ca^{2+}]_i$ increased only to 8 ± 1% ($n = 92$) above basal upon Fe^{2+} ions application for 30 min. Such an α-TP pretreatment completely masked the inhibitory effect of CPZ on Fe^{2+} ion-mediated $[Ca^{2+}]_i$ increase; it only reached 6 ± 1% above basal after a 30 min exposure to 50 μM $FeSO_4$ in the presence of 10 μM CPZ in pre-α-TP cells (Fig. 3).

3.3 Effect of CPZ on capacitive calcium entry in hippocampal neurons

Capacitive calcium entry (CCE) following metabotropic glutamate stimulation within neurons was measured by fura-2 fluorescence imaging. We have previously demonstrated that αTP-pretreatment strongly inhibited CCE [16]. CCE was obtained by first stimulating cells for 3 min with 30 μM glutamate in an extracellular buffer devoid of calcium. Under these conditions, a transient peak of $[Ca^{2+}]_i$ was observed corresponding to the release of calcium from intracellular stores (Fig. 4). Following this, the extracellular medium was then replaced by a buffer containing 2 mM $CaCl_2$. This produced a large rise in $[Ca^{2+}]_i$, known as CCE, since it likely serves to refilling intracellular stores within neurons. In the presence of 10 μM CPZ, this CCE was largely reduced. Indeed, the maximal $[Ca^{2+}]_i$ reached in the presence of CPZ was 127 ± 2% of basal $[Ca^{2+}]_i$ ($n = 249$), as compared with 151 ± 2% ($n = 453$) without CPZ.

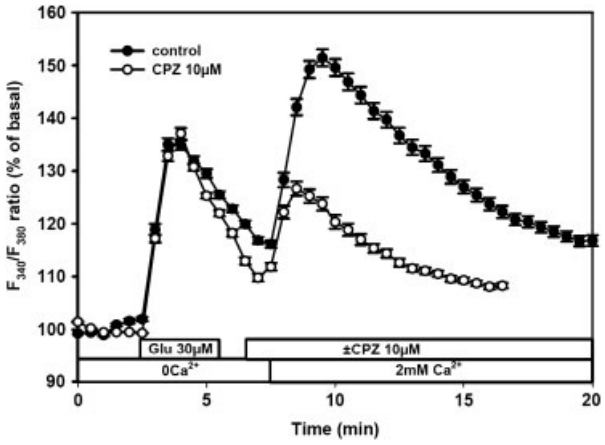


Figure 4. CPZ inhibits CCE in hippocampal neurons. Effect of TRPV1 channel blocker CPZ on the CCE occurring after metabotropic glutamate receptors stimulation. Cells were rinsed with Ca^{2+}-free medium and glutamate (Glu, 30 μM) was then applied for 3 min. Glu was washed off for either 2 min in Ca^{2+}-free medium or 1 min in Ca^{2+}-free medium, followed by 1 min in Ca^{2+}-free medium containing CPZ (10 μM). Ca^{2+}-rich medium, supplemented or not with CPZ (10 μM), was then applied to reveal CCE. On graphs, data are expressed as average ± SE of F340/F380 ratio normalized to basal F340/F380 ratio.

3.4 Effect of CPZ on *Ih* currents

An effect of CPZ on *Ih* currents associated with its neuroprotective activity was previously reported [27]. Such *Ih* currents could be measured upon hyperpolarisation of neurons from −60 to −120 mV (Fig. 5A). These currents were inhibited by both 20 μM CPZ and 10 μM ZD 7288, the prototypic antagonist of channels supporting *Ih* currents, as well by 5 mM CsCl, an unselective blocker of potassium channels. Amplitudes of *Ih* currents were decreased to 63 ± 6 ($n = 18$) and 74 ± 8% of control by CPZ and ZD 7288, respectively. In α-TP-pretreated neurons, *Ih* current amplitude was similar to that measured in control cells. Moreover, under this condition, inhibitions induced by CPZ or ZD 7288 were not significantly different from those obtained in control neurons.

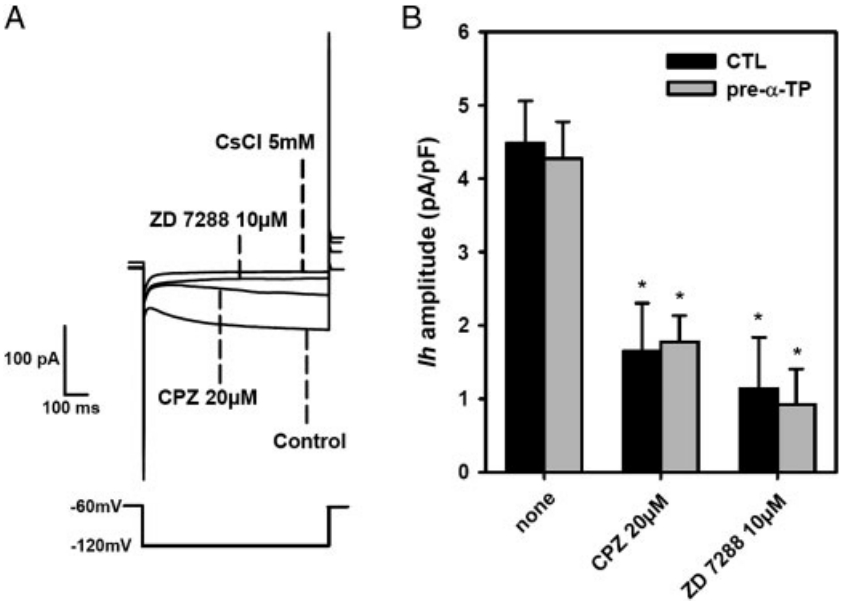


Figure 5. CPZ blocks *Ih* channels in cultured hippocampal neurons. (A) Sample traces from whole-cell patch-clamp recording of *Ih* current (hyperpolarization-activated cationic current). These currents were induced by hyperpolarizing cells from −60 to −120 mV for 1 s. CPZ (20 μM) and the *Ih* specific blocker ZD 7288 (10 μM) were used for pharmacological characterization of these currents. Drugs were washed between each application. Cesium chloride (CsCl, 5 mM), known to completely abolish potassium currents, was applied at the end of the recordings. (B) Recapitulative graph plotting *Ih* amplitudes obtained in the presence of either CPZ or ZD 7288 in control ($n = 18$ for each condition) and α-TP-pretreated neurons ($n = 14$ for each condition). Amplitudes of *Ih* were calculated by subtracting amplitudes of the current to that obtained in the presence of CsCl. They were then normalized to the respective cell capacitances. Data are presented as mean ± SEM. $^*p < 0.05$ *versus* respective control; $^\sharp p < 0.05$ *versus* data obtained in control cells.

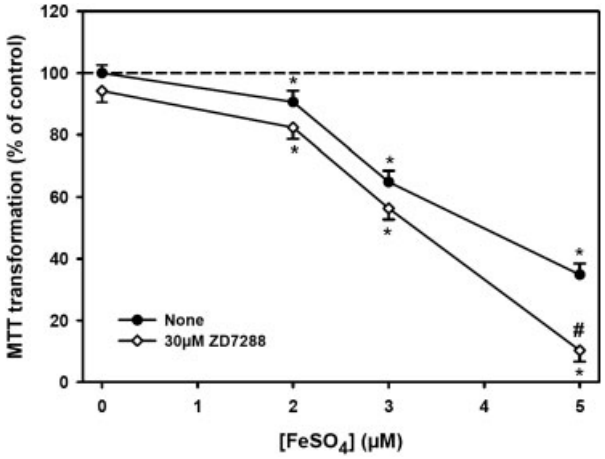


Figure 6. The *Ih* blocker, ZD 7288, was not neuroprotective against the Fe^{2+} ion-elicited toxicity. ZD 7288 (30 μM) was coapplied with different concentrations of $FeSO_4$. Cell survival was measured 24 h later by dosage of MTT transformation. On the graphs, data are means ± SEM of a representative experiment performed in triplicate. They are expressed as percentages of MTT transformation obtained in control culture without any treatment. $^{*}p<0.05$ *versus* $FeSO_4$ alone; $^{\sharp}p<0.05$ *versus* respective control.

3.5 Effect of ZD 7288 on the oxidative stress-induced neuronal death

The *Ih* current inhibitor, ZD 7288, was further tested for its ability to prevent the Fe^{2+} ion-induced toxicity. In fact, 30 μM ZD 7288 not only failed to prevent neuronal death but even slightly enhanced cell damages obtained elicited by $FeSO_4$. For instance, 5 μM $FeSO_4$ induced a 65 ± 4% decrease in cell survival in the absence of ZD 7288, as compared with a 90 ± 4% decrease in its presence (Fig. 6). It should be noted that, *per se* 30 μM ZD 7288 did not significantly affect neuron survival.

3.6 Effects of TRPV1 antagonists on spontaneous glutamatergic transmission

The direct activation of TRPV1 receptors with selective agonists such as capsaicin or resiniferatoxin did not induce any whole-cell currents in cultured hippocampal neurons (data not shown) as compared to dorsal root ganglion neurons that exhibit an exquisite sensitivity to TRPV1 agonists [28]. In some experimental models, TRPV1 receptors regulate the neurotransmission mediated by glutamate [29–31]. We, thus, decided to test whether TRPV1 channel blockade with either CPZ or I-RTX could alter spontaneous excitatory synaptic activity by recording sEPSC using whole-cell patch-clamp in hippocampal neurons. The application of CPZ (5–20 μM) or of I-RTX (0.3–3 μM) actually decreased the frequency of sEPSC in a concentration-dependent manner (Fig. 7A and 7B). Interestingly, in α-TP-pretreated neurons, both substances lost their ability to inhibit spontaneous excitatory synaptic activity. In control neurons, CPZ (20 μM) induced a 78 ± 12% ($n = 7$) decrease in bursting frequency, while in α-TP-pretreated neurons CPZ decreased this activity only by 23 ± 15% ($n = 7$) (Fig. 7A). Similarly, in the presence of 3 μM I-RTX, bursting frequency was decreased by 35 ± 4% in control neurons ($n = 7$), while it was fully inactive in α-TP-pretreated neurons ($n = 7$, Fig. 7B). Any significant effect of either CPZ or I-RTX on sEPSC amplitudes was observed, neither in control nor in α-TP-pretreated neurons.

4 Discussion

We previously demonstrated that a pretreatment of hippocampal neurons with a low concentration of α-TP renders them durably resistant to an oxidative stress evoked by Fe^{2+} ions [12]. This resistance is related to a decreased function of a channel belonging to the TRP channel family and responsible for the calcium overload triggered by Fe^{2+} ions [16]. In this article, we attempted to further identify the subtype of TRP channels involved. According to our previous observations, these channels have to fulfill at least the following four requirements: (i) they must be involved in the entry of Ca^{2+} within neurons elicited by Fe^{2+} ions; (ii) their blockade must protect neurons against oxidative insult; (iii) they should participate in the mechanisms of CCE in hippocampal neurons; and (iv) their function must be negatively modulated by the α-TP pretreatment of hippocampal neurons. Here, we demonstrate that the TRPV1 channel effectively carries out all these four criteria.

Indeed, we show here that the pharmacological blockade of TRPV1 channels with selective antagonists, *i.e.* CPZ or I-RTX, can prevent cell damage triggered by exposing neuronal cells to Fe^{2+} ions. The neuroprotective effect of TRPV1 channel blockade suggests that these channels play a critical role in the mediation of the Ca^{2+} influx associated with neuronal death. Actually, the TRV1 channel antagonist CPZ also largely reduces the intracellular Ca^{2+} overload triggered by Fe^{2+} ions, as we previously observed with broad-range TRP channel blockers. However, CPZ may also act on molecular targets other than TRPV1. For instance, it was reported that the CPZ-elicited neuroprotection against injury triggered by oxygen glucose deprivation in hippocampal slices occurs through the action of this molecule on *Ih* channels [27]. We show here that CPZ actually inhibits *Ih* currents in hippocampal neurons, just like ZD 7288-a well-known inhibitor of these channels. However, ZD 7288 is not able to prevent Fe^{2+} ion toxicity and even tends to amplify it, excluding the participation of the blockade of *Ih* currents in the CPZ-induced neuroprotection in our experimental model. Moreover, another more specific TRPV1 antagonist, I-RTX, structurally unrelated to CPZ also protects neurons against oxidative stress, which confirms

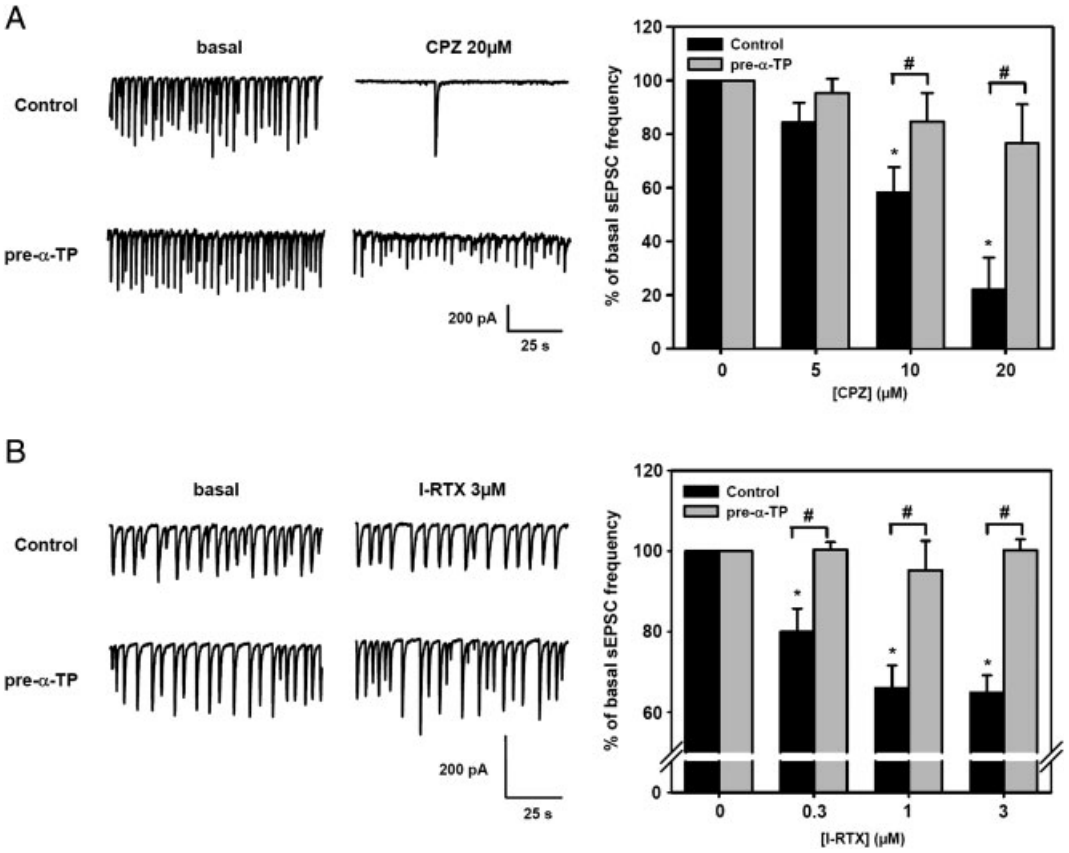


Figure 7. α-TP pretreatment blunts CPZ- and I-RTX--mediated inhibition of the excitatory synaptic bursting activity. Spontaneous excitatory synaptic transmission was monitored in Mg^{2+}-free extracellular medium supplemented with 10 μM glycine in hippocampal neurons voltage clamped at −60 mV. Under these experimental conditions, spontaneous excitatory postsynaptic currents (sEPSC) were mainly mediated by NMDA receptor activation. (A) Effects of increasing concentrations of CPZ (from 5 to 20 μM) on bursting activity. On the left, representative samples illustrating the effect of 20 μM CPZ on sEPSC recorded either in control or in α-TP-pretreated neurons are shown. On the right, recapitulative graph of average sEPSC frequencies is plotted as a function of CPZ concentrations in either control or pre-α-TP neurons. (B) Effects of increasing concentrations of I-RTX (from 0.3 to 3 μM) on bursting activity. On the left, representative samples illustrating the effect of 3 μM I-RTX on sEPSC recorded either in control or in α-TP-pretreated neurons are shown. On the right, recapitulative graph of average sEPSC frequencies are plotted as a function of CPZ concentrations in either control or pre-α-TP neurons. For the generation of recapitulative graphs, the calculation of bursting frequency was performed on a 5-min time window before and during CPZ or I-RTX application. Frequencies were further normalized to their respective basal frequency obtained without CPZ or I-RTX. *Per se*, the α-TP pretreatment did not significantly modify the basal frequency of sEPSC (0.33 ± 0.03 and 0.32 ± 0.03 Hz in control and α-TP-pretreated neurons, respectively). Data are mean ± SEM. $^{*}P < 0.05$ *versus* respecting basal bursting frequency; $\sharp p < 0.05$ between control and α-TP-pretreated neurons.

that the neuroprotection observed in the presence of CPZ is actually due to the blockade of TRPV1 channels.

Thus, protection elicited by TRPV1 antagonist is linked to an inhibition of the exaggerated Ca^{2+} influx occurring during oxidative insult. Therefore, we can hypothesize that TRPV1 channels are overactivated by an oxidative stress and thus mediate the lethal intracellular Ca^{2+} overload. It has been previously evidenced that other members of the TRP channel family, *i.e.* TRPM2 and TRPM7 channels, could be strongly activated under oxidative conditions and then mediate cell death in cortical neuronal cultures [17, 32]. Our data further extent the panel of TRP channels involved in the regulation of neuronal death under oxidative stress. Interestingly, an increase in TRPV1 function has also been recently described in pulmonary arterial smooth muscle cells following chronic hypoxia [33].

Hippocampal neuron pretreatment with α-TP induces a large inhibition of the CCE that follows metabotropic glutamate receptor stimulation. As we previously showed for broad-range TRP channel blockers, a similar inhibition of the CCE is found upon the application of CPZ. Thus, calcium influx through TRPV1 channel can participate in the CCE within hippocampal neurons. To our knowledge, this is the first evidence for a role of TRPV1 channels as store-operated channels in neurons. Strikingly, in pulmonary arterial smooth muscle cells, where TRPV1 are overactivated upon chronic hypoxia, these channels also support CCE [33].

The pretreatment by α-TP fully occludes the effects of CPZ both on Fe^{2+}-induced $[Ca^{2+}]_i$ increase and on the CCE. This indicates that to some extent, α-TP pretreatment decreases the activity of TRPV1 channels, consistent with our hypothesis that down-regulation of TRPV1 channels activity by α-TP pretreatment should be responsible for the long-lasting neuroprotection of neurons against oxidative stress. In fact, measuring the regulation of spontaneous excitatory synaptic activity by TRPV1 antagonists in either control or α-TP-pretreated neurons also confirms this. Indeed, both CPZ and I-RTX by themselves depress excitatory spontaneous synaptic transmission, and following α-TP pretreatment, there is a marked decrease in the efficiency of these TRPV1 antagonists. These data strongly suggest that α-TP pretreatment induces a down-regulation of a tonic activity of TRPV1 channels. Indeed, the fact that TRPV1 channel blockade with antagonists depresses *per se* excitatory synaptic transmission strongly suggests that, under control conditions, TRPV1 channels are tonically activated, resulting in a chronic activation of spontaneous excitatory synaptic transmission. This tonic activity of TRPV1 channel is apparently lost in α-TP-pretreated neurons, as evidenced by the lack of effect of TRPV1 antagonists on sEPSC under these conditions. The tonic activation of TRPV1 channels observed in control cultures may result either from the presence of endogenous ligand(s), *i.e.* endovanilloids/endocannabinoids, as it has been described in neurons of the rostral ventromedial medulla [34] or from a constitutive open state of these channels in hippocampal neurons. In this context, activation of TRPV1 may depend on the phosphorylation of the channel [35, 36]. Indeed, several reports indicate that phosphorylations by protein kinase A, C, or calcium calmodulin kinase 2 sensitize the channel and potentiate its opening under agonist stimulation, while calcineurin-mediated dephosphorylation desensitizes TRPV1 responses [36].

Conversely, to regulate negatively TRPV1 channels activity, α-TP pretreatment may target various mechanisms: Most simply, a decreased expression could account for a reduced activity of these channels. However, preliminary Western blot analyses did not evidence any significant change in the global expression of these channels in α-TP-pretreated neurons as compared with control. Alternatively, many other mechanisms—potentially related to each other—could explain such a decrease in TRPV1 channel activity in pre-α-TP cells, which are listed as follows: (i) a modification of membrane targeting of the channels; (ii) interactions with specific partner(s), comprising other TRP channel sub-units; (iii) post-translational modifications such as phosphorylation; and (iv) the modulation of endogenous ligand availability.

For instance, an altered traffic of the channel to the membrane could be triggered by α-TP pretreatment. Interestingly, it was recently demonstrated that oxidative conditions could facilitate membrane insertion of TRPV1 channels [37]. Moreover, a substantial number of reports suggest that TRPV1 channel activity can be modulated by several effectors [36]. As we previously indicated, phosphorylation on Ser/Thr residues is critical for their function [35, 38–40]. Indeed, PKC-dependent phosphorylation has been shown to facilitate TRPV1 channels activity and activation, especially in sensory neurons [41–44]. Interestingly, α-TP has been known for long as an inhibitor of PKC activity [45]. Therefore, in one hand, a defect in PKC-dependent phosphorylation could explain such a reduced activity of TRPV1 following α-TP pretreatment. On the other hand, the fact that α-TP pretreatment decreases the effect of TRPV1 antagonists could result from depletion in endogenous agonists of the channel, such as endovanilloids/endocannabinoids. In fact, PKC is also able to promote the synthesis of endovanilloids/endocannabinoids [46, 47] and this could participate in the neuroprotective effects of α-TP pretreatment. Thus, the decreased activity of TRPV1 channel antagonists could be a consequence of a depletion of endogenous agonists in α-TP-pretreated neurons *via* the PKC inhibition.

Although some studies have shown beneficial effects of an increase in the dietary intake of vitamin E, its action is still a matter of controversy rendering its use debatable, especially for the treatment of Alzheimer's disease [5]. Vitamin E may modulate a plethora of cellular/biochemical mechanisms, some of them unrelated to its antioxidant activity [6]. This may, thus, lead to the occurrence of 'side-effects' when chronically used. The accurate search for the targets modulated by vitamin E under specific conditions, such as oxidative stress, could help for designing modulators of these targets. For instance, from our data, one could propose that modulators of TRPV1 channels could exhibit a neuroprotective action comparable to that elicited by vitamin E with a more selective action than vitamin E itself. Interestingly, TRPV1 channels are modulated by vanilloids and endovanilloids/endocannabinoids, a large family of lipid compounds [46, 47]. Hence, we can hypothesize that synaptic neurotransmission in hippocampal neurons is regulated by integration of responses induced by both endogenous and exogenous lipids, including vitamin E itself; these lipids could then endow neurons with new properties such as resistance against oxidative insults.

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5 References

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