



Inhibitions of anandamide transport and FAAH synthesis decrease apoptosis and oxidative stress through inhibition of TRPV1 channel in an in vitro seizure model

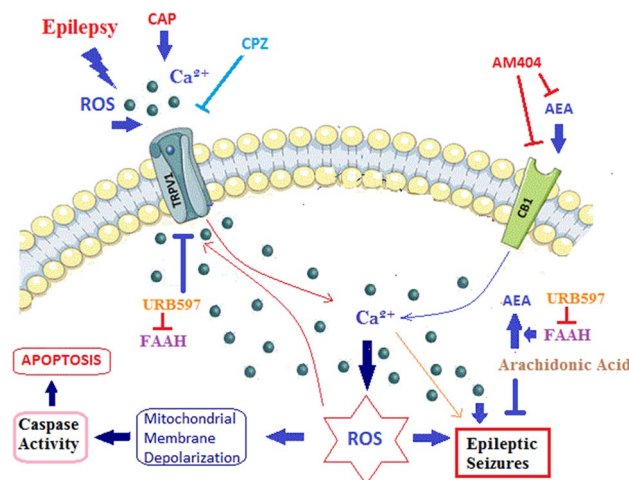
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Abstract

The expression level of TRPV1 is high in hippocampus which is a main epileptic area in the brain. In addition to the actions of capsaicin (CAP) and reactive oxygen species (ROS), the TRPV1 channel is activated in neurons by endogenous cannabinoid, anandamide (AEA). In the current study, we investigated the role of inhibitors of TRPV1 (capsazepine, CPZ), AEA transport (AM404), and FAAH (URB597) on the modulation of Ca^{2+} entry, apoptosis, and oxidative stress in in vitro seizure-induced rat hippocampus and human glioblastoma (DBTRG) cell line. The seizure was induced in the hippocampal and DBTRG neurons using in vitro 4-aminopyridine (4-AP) to trigger a seizure-like activity model. CPZ and AM404 were fully effective in reversing 4-AP-induced intracellular free Ca^{2+} concentration of the hippocampus and TRPV1 current density of DBTRG. However, AEA and CAP did not activate TRPV1 in the URB597-treated neurons. Hence, we observed TRPV1 blocker effects of URB597 in the DBTRG neurons. In addition, the AM404 and CPZ treatments decreased intracellular ROS production, mitochondrial membrane depolarization, apoptosis, caspases 3 and 9 values in the hippocampus. In conclusion, the results indicate that inhibition of AEA transport, FAAH synthesis, and TRPV1 activity can result in remarkable neuroprotective effects in the epileptic neurons.

Graphical abstract Possible molecular pathways of involvement of capsazepine (CPZ) and AM404 in anandamide and capsaicin (CAP)-induced apoptosis, oxidative stress, and Ca^{2+} accumulation through TRPV1 channel in the seizure-induced rat hippocampus and human glioblastoma neurons. The TRPV1 channel is activated by different stimuli including reactive oxygen species (ROS), anandamide (AEA), and CAP and it is blocked by capsazepine (CPZ). Cannabinoid receptor type 1 (CB1) is also activated by AEA. The AEA levels in cytosol are decreased by fatty acid amide hydrolase (FAAH) enzyme. Inhibition of FAAH through URB597 induces stimulation of CB1 receptor through accumulation AEA. URB597 acts antiepileptic effects through inhibition of TRPV1. Overloaded Ca^{2+} concentration of mitochondria can induce an apoptotic program by stimulating the release of apoptosis-promoting factors such as caspases 3 and caspase 9 by generating ROS due to respiratory chain damage. AM404 and CPZ reduce TRPV1 channel activation and Ca^{2+} entry in the in vitro 4-AP seizure model-induced hippocampal and glioblastoma neurons.



Keywords Anandamide · Apoptosis · Epilepsy · FAAH inhibition · TRPV1 · Oxidative stress

Abbreviations

[Ca ²⁺] _i	Intracellular free calcium ion
4-AP	4-Aminopyridine
aCSF	Artificial cerebrospinal fluid
AEA	Anandamide
CAP	Capsaicin
CPZ	Capsazepine
CRR	Cannabinoid reuptake
DBTRG	Denver Brain Tumor Research Group
EGTA	Ethylene glycol-bis[2-aminoethyl-ether]- <i>N,N,N,N</i> -tetraacetic acid
FAAH	Fatty acid amide hydrolase
HBSS	Hank's buffered salt solution
IRTX	5'-Iodoresiniferatoxin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
TRP	Transient receptor potential
TRP	Transient receptor potential
TRPV1	Transient receptor potential vanilloid 1
VGCC	Voltage-gated calcium channels

Introduction

Epilepsy is characterized as an acute transient complex neurobehavioral disorder affecting approximately 50 million of the population worldwide [1]. It is well known that a feature of epileptic activity is excessive synchronous neuronal activity in the brain. The *in vitro* 4-aminopyridine (4-AP) model has been widely used during the last three decades to identify the mechanisms underlying the synchronization of hippocampal neuronal networks in epilepsy [2, 3]. Accumulated

overload of Ca²⁺ and Na⁺ concentrations through activation of cation channels in the hippocampus has an important role in the epileptogenesis and most of antiepileptic drugs have been used for treating epilepsy through modulation of the cation channel activities including transient receptor potential (TRP) channels [4–6]. Pathophysiological functions including cell viability, oxidative stress, apoptosis, and hippocampal activations are regulated by the intracellular Ca²⁺ concentration [7, 8]. The role of cellular and molecular pathophysiology of epileptic injury-induced hippocampal overloaded Ca²⁺ influx and apoptosis remains unclear.

The TRP vanilloid 1 (TRPV1) is a member of the TRP family [9, 10]. When TRPV1 channel is activated, it leads to augmented permeability of Na⁺ and Ca²⁺, increasing neuronal excitability [6]. Capsaicin as a pungent extract of hot chili pepper activates a subset of hippocampal neurons by binding to TRPV1 channel [6, 11]. In addition, excessive production of reactive oxygen species (ROS) has an important role on activation of TRPV1 channels in neurons due to the content of highly oxidizable cysteine groups possessed by the channels [9, 11]. Hippocampal CA1 and CA3 regions have important role on etiology of epilepsy and expression levels of TRPV1 channels are high in the regions [12]. Accumulating interest in the therapeutic potential of TRPV1 provides support for the hypothesis that TRPV1 channel inhibition likely underlies many of the benefits associated with decreased epilepsy [4, 5]. This effect should be clarified in the *in vitro* 4-AP seizure model.

Anandamide (*N*-arachidonoyl-ethanolamide, AEA) is well known as an endogenous cannabinoid neuromodulator [13, 14], which shows considerable structural similarity to capsaicin [1]. It was the first identified endocannabinoid,

acting on at least two G-protein-coupled cannabinoid receptors namely CB1 and CB2 [15]. Hippocampus has high concentration of AEA [16]. Fatty acid amide hydrolase (FAAH) is a main enzyme in cytosol for decreasing AEA levels and its inhibition through URB597 induces stimulation of CB1 receptor through accumulation AEA [17, 18]. In addition, AEA induced URB597-independent effects in neuroblastoma cells and its low-dose injection into rat brain induced decreased thermal nociception via TRPV1 inhibition [18]. Some cation channels including TRPV1 channels are activated in the central nervous system by AEA and FAAH inhibitors [19–21]. Positive correlation between AEA-induced seizure and TRPV1 activation was also reported [22]. We have recently reported that TRPV1-specific antagonists, capsazepine (CPZ) and 5'-iodoresiniferatoxin (IRTX), have protective effects on epileptic seizures and on Ca^{2+} entry in the hippocampal neuron of the epilepsy-induced adult rat [6, 23]. Thus, the dual blockade of TRPV1 and AEA transport could represent an interesting strategy in epilepsy for the development of new pharmacological strategies. *N*-Arachidonoylamino-phenol (AM404) is an active metabolite of paracetamol and an AEA reuptake inhibitor [19]. Limited results indicated in hippocampus that AM404 is also a TRPV1 channel agonist and it has no adverse effects on the TRPV1 channel in the neuron [24, 25]. AM404 prevents transport of AEA through cell membranes [19] and it has been used in molecular and behavioral investigations of the endocannabinoid system. AM404 prevents pain and behavior through modulation of voltage-gated calcium channel (VGCC) activity and apoptotic pathways in a rat epileptic model [26]. However, it is still unknown whether AEA and FAAH regulate neuronal Ca^{2+} concentration and apoptosis of hippocampus and glioblastoma cells through modulating TRPV1 channels in the in vitro 4-AP seizure model.

We investigated the inhibitions of AEA and FAAH but activation of TRPV1, and compared these with the effects of the TRPV1 channel agonist, capsaicin, in the in vitro 4-AP seizure-induced cell line and rat hippocampus models.

Materials and methods

Animals

Forty-two (8–10 weeks) male adult Wistar albino rats were used in the current study. The animals were housed in a room with a temperature of $22 \pm 2^\circ\text{C}$ and humidity at 60%. The environment included light control with a 12-h cycle starting at 08:00 h. The rats were fed with commercial food and allowed to drink water ad libitum. The study was approved by the Local Experimental Animal Ethical Committee of Suleyman Demirel University (SDU) (Protocol number: 20.02.2015-06). The animals were maintained

and used according to the Animal Welfare Act and the Guide for the Care and Use of Laboratory.

Preparation of hippocampal neuron samples

The animals were killed by cervical dislocation in accordance with SDU Experimental Animal legislation. Brains were rapidly removed and placed in an ice cold solution consisting of (in mM) 86 NaCl, 3 KCl, 4 MgCl_2 , 1 NaH_2PO_4 , 75 sucrose, 25 glucose, 1 CaCl_2 , and 25 NaHCO_3 . Primary cultures of rat hippocampal neurons were prepared as previously described [23]. Hippocampal tissue was dissected and placed in cold Hank's buffered salt solution (HBSS) prior to mechanical dissociation by trituration and incubated for 30 min with trypsin and mixed every 10 min. It was centrifuged (at $500 \times g$ for 5 min) and the supernatant was discarded and replaced by Hanks' solution twice more and then used in assays. The neurons were plated at a density of $< 1 \times 10^6$ cells ml^{-1} on 35-mm culture dishes. These slices were placed in artificial cerebrospinal fluid (aCSF) containing (in mM) 120 NaCl, 4.5 KCl, 1 MgCl_2 , 10 glucose, 1 CaCl_2 , 1.25 NaH_2PO_4 , and 26 NaHCO_3 at 33°C for 30 min to recover; afterward, they remained in aCSF at room temperature until used in experiments. All solutions were maintained at pH 7.4 by continuous bubbling with 95% O_2 and 5% CO_2 .

Cell culture of DBTRG neuroblastoma

We have recently observed high levels of TRPV1 channel expression in the Denver Brain Tumor Research Group (DBTRG) cell line (submitted data). The neuroblastoma cells have been mostly used in TRPV1, FAAH, and AEA studies for investigation of neurological disease including epilepsy and invasion of neuroblastoma cells [18, 27]. Hence, the cell was used in this study. The cell line was originally obtained from 'The Leibniz Institute—German Collection of Microorganisms and Cell Cultures' Cell Lines Bank (Braunschweig, Germany). The cells were cultured in RPMI 1640 medium supplemented with fetal bovine serum in a humidified incubator at 37°C , 5% CO_2 , and 95% air. The cells were counted daily by removing a small volume from the tissue culture flask (filter cap, sterile, 250 ml, 75 cm^2), diluting it with an equal volume of trypan blue (0.4%), and tallying viable cells (trypan blue excluding) with a cell counter (Casy Modell TT, Roche, Germany). Cultures were maintained as a suspension without shaking or stirring at a density of 1×10^6 cells per ml by dilution with fresh media. Cultures were transferred once a week. The cells were used for the electrophysiological analyses.

Study groups

Hippocampal neuron of each animal and DBTRG neuroblastoma cell lines were divided into seven groups as follows:

- I. Control group ($n=6$). The neurons of the control group were incubated (37 °C and 5% CO₂) for 30 min with RPMI medium and they were stimulated by capsaicin (10 μM) [6, 23].
- II. 4-AP group ($n=6$): The hippocampal neurons were incubated with 0.1 mM 4-AP (Sigma Chemical Co., St. Louis, MO, USA) (37 °C, 5% CO₂) for 60 min [28] and then stimulated by capsaicin (10 μM).
- III. AEA group ($n=6$): The neurons were incubated with 10 μM AEA (37 °C, 5% CO₂) for 20 min [13] and then stimulated by CAP (0.1 mM).
- IV. AM404 group ($n=6$): The neurons were incubated with AM404 (1 μM for 20 min) [26]. Then they were stimulated by capsaicin (10 μM).
- V. AM404 + 4-AP group ($n=6$): The neurons were incubated with AM404 (1 μM for 20 min). Then they were incubated with 0.1 mM 4-AP (37 °C, 5% CO₂) before stimulation via capsaicin (10 μM) and AEA (10 μM).
- VI. CPZ group ($n=6$): The neurons were incubated with CPZ (0.1 mM for 30 min) [23]. Then they were stimulated by capsaicin (10 μM).
- VII. CPZ + 4-AP + AEA ($n=6$): The neurons were incubated with CPZ (0.1 mM for 30 min). Then they were incubated with 0.1 mM 4-AP for 60 min and 10 μM AM404 (37 °C, 5% CO₂) for 20 min before stimulation via capsaicin (10 μM).
- VIII. URB597 group ($n=6$): The neurons were incubated with a FAAH inhibitor (URB597 and 1 μM for 60 min) [29] before stimulation via capsaicin (10 μM) and AEA (10 μM).
- IX. 4-AP + URB597 group ($n=6$): The neurons were incubated with a FAAH inhibitor (URB597 and 1 μM for 60 min) [29]. Then they were incubated with 0.1 mM 4-AP for 60 min before stimulation via capsaicin (10 μM) and AEA (10 μM).

Measurement of intracellular free calcium ([Ca²⁺]_i) concentration

The [Ca²⁺]_i was measured with the fluorescent indicator Fura-2-AM [30]. To measure, cells were trypsin-digested, allowed to sediment, and re-suspended in HEPES-buffered medium [20 mM HEPES (pH 7.4), 10 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.5 mM CaCl₂, 25 mM NaHCO₃, 15 mM glucose, and 0.1% bovine serum albumin (fatty acid free)]. Then they were incubated with 5 μM fura-2AM (Calbiochem, Darmstadt, Germany) in a

water bath (37 °C) for 45 min. Fluorescence changes were recorded on a spectrofluorometer (Carry Eclipse, Varian Inc, Sydney, Australia) from 2 ml aliquots of magnetically stirred cellular suspensions at 37 °C. Excitation wavelengths in the analyses were 340 and 380 nm and the emission wavelength was 505 nm. Changes in the integral of the Ca²⁺ response were monitored using the Fura-2 340/380 nm fluorescence ratio and were calibrated according to the method of Grynkiewicz [31].

Ca²⁺ entry in the hippocampal neurons was estimated using the integral of the rise in [Ca²⁺]_i concentration for the first 170 s after adding capsaicin (10 μM). Ca²⁺ entry is expressed in nanomolar quantities by taking a sample every second as previously described [32, 33].

Electrophysiology

In addition to oxidative stress and capsaicin, TRPV1 channel is activated by hot temperature [11]. Therefore, whole-cell voltage clamp recording in DBTRG neuroblastoma was performed from in room temperature at 21–23 °C (EPC10 patch-clamp set, HEKA, Lamprecht, Germany). Resistances of the recording electrodes were adjusted to about 3–7 MΩ by a puller (PC-10 Narishige International Limited, London, UK). We used standard extracellular bath and pipette solutions as described in previous studies [6, 23]. Holding potential of the patch-clamp analyses in the cells was –60 mV. Voltage clamp technique was used in the analyses, and current–voltage (*I*–*V*) relationships were obtained from voltage ramps from –90 to +60 mV applied over 200 ms.

In the experiments, TRPV1 was extracellularly gated by capsaicin (10 μM), and the channels were extracellularly blocked by CPZ (0.1 mM). For the analysis, the maximal current amplitude (pA) in a DBTRG neuron was divided by the cell capacitance (pF), a measure of the cell surface. The results in the patch-clamp experiments are the current density (pA/pF).

Intracellular reactive oxygen species (ROS) production measurement

Rhodamine 123 (Rh-123) (cell-permanent, uncharged, and green-fluorescent dye) was used to monitor the intracellular ROS production in the neurons [34]. The hippocampal neurons (10⁶ neurons/ml) were washed with serum-free RPMI-1640 medium and incubated with 0.02 mM DHR-123 at 37 °C for 25 min [33]. The fluorescence intensity of Rh 123 was measured (excitation: 488 nm and emission: 543 nm) in an automatic microplate reader (Infinite pro200; Tecan Austria GmbH, Groedig, Austria). Data are presented as fold-increase over the pretreatment level (experimental/control).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. JC-1 cationic dye was used for determination of the mitochondrial membrane potential ($\Delta\Psi_m$) [33]. The hippocampal neurons were incubated with JC-1 at 37 °C for 45 min. JC-1 fluorescence was measured by a single excitation wavelength (488 nm) with dual emission, green (520 nm) and red (596 nm), using the microplate reader (Infinite pro200). The value of $\Delta\Psi_m$ is calculated as the ratio of red to green fluorescence. The relative transmembrane potential was expressed as a percentage of $\Delta\Psi_m$ relative to vehicle control (fold-increase).

Assay for apoptosis, caspase 3 and caspase 9 activities

The apoptosis assay was performed using a commercial kit according to the instructions provided by Biocolor Ltd. (Northern Ireland) and elsewhere [6]. The APOPercentage dye is actively transported into cells, staining apoptotic cells red, thus allowing detection of apoptosis by a spectrophotometer (Shimadzu-UV 1800, Kyoto, Japan).

The determinations of caspase 3 and caspase 9 activities in hippocampus were based on methods previously reported [32, 33] with minor modifications. Stimulated or resting cells were washed once with PBS. Fifteen μ l of the neuron suspension (10^6 neurons/ml) were added to the microplate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer that was composed of 100 mM HEPES, pH 7.25, 10% sucrose, 0.1% 3-[(3 cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM DTT, 0.001% nonidet-P-40 substitute (NP40) and 0.04 ml of caspase 3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin and Bachem, Bubendorf, Switzerland) or 0.1 M 2-(*N*-morpholino) ethanesulfonic acid hydrate (MES hydrate), pH 6.5, 10% polyethylene glycol (PEG), 0.1% CHAPS, 5 mM DTT, 0.001% NP40, and 0.1 mM of caspase 9 substrate (His-Asp-7-amino-4-methylcoumarin and Bachem, Bubendorf, Switzerland). Substrate cleavage was measured with the microplate reader with excitation wavelength of 360 nm and emission at 460 nm. The data were calculated as fluorescence units/mg protein and presented as fold-increase over the pretreatment level (experimental/control).

Statistical analyses

All results were expressed as means $\pm$ standard deviation (SD) and *p* values less than 0.05 were regarded as significant. To determine the effect of treatment, data were analyzed using ANOVA. Presence of significance was assessed

with an LSD test. The significance values in six groups were assessed with an unpaired Mann–Whitney *U* test. The SPSS statistical program was used to analyze the data (version 17.0 software, SPSS Inc. Chicago, IL, USA).

Results

The effects of AM404 and CPZ on $[Ca^{2+}]_i$ accumulation through TRPV1 activity in the hippocampus of rats

CPZ is a specific TRPV1 channel antagonist, although AM404 is an AEA transport blocker [11, 26, 35, 36]. This study used it in the presence of AEA- and capsaicin (CAP)-induced Ca^{2+} entry in order to identify the TRPV1 and AEA transport involved in Ca^{2+} accumulation via TRPV1 channels. As shown in Fig. 1A, B, the $[Ca^{2+}]_i$ concentration in hippocampal neurons was significantly ($p < 0.001$) higher in the 4-AP + CAP and AEA + CAP groups compared to the control animals. However, the $[Ca^{2+}]_i$ concentration was also markedly ($p < 0.05$) lower in the AEA + CAP group when compared to the 4-AP + CAP group. We also observed a modulator role for CPZ and AM404 on the $[Ca^{2+}]_i$ concentration in the neurons. Its concentration was significantly ($p < 0.001$) lower in the AM404 + CAP, AM404 + 4-AP + CAP, CPZ + 4-AP + CAP, and CPZ + 4-AP + AEA + CAP ($p < 0.001$) groups than in the 4-AP + CAP and AEA + CAP groups. In addition, the $[Ca^{2+}]_i$ concentration in the neurons was markedly ($p < 0.05$) lower in CPZ + 4-AP + CAP and CPZ + 4-AP + AEA + CAP groups than in the AM404 + CAP, AM404 + 4-AP + CAP groups. Hence, the role of TRPV1 channels on $[Ca^{2+}]_i$ concentration in the experimental seizure model seems more important than in inhibition of AEA transport across the cell membrane.

The effects of AM404, CPZ, and URB597 on TRPV1 current densities in the DBTRG cells

Our patch-clamp sets are suitable for single-cell analyses instead of tissue slices. Diameter of tip of patch pipette is around 1–5 μ m and adult hippocampal neurons are sucked in the whole-cell configuration by the tip of path pipette. As described above, glioblastoma cells, including DBTRG, have been mostly used in TRPV1, FAAH, and AEA studies [18, 27]. Therefore, we used the DBTRG instead of the small hippocampal neurons in the patch-clamp experiments. The presence of TRPV1 channels were tested by CAP and CPZ. The current densities of CAP were significantly ($p < 0.001$) higher in control + CAP cells than in control cells (without CAP stimulation), although they were decreased in 4-AP + CPZ + CAP group by CPZ treatment ($p < 0.001$)

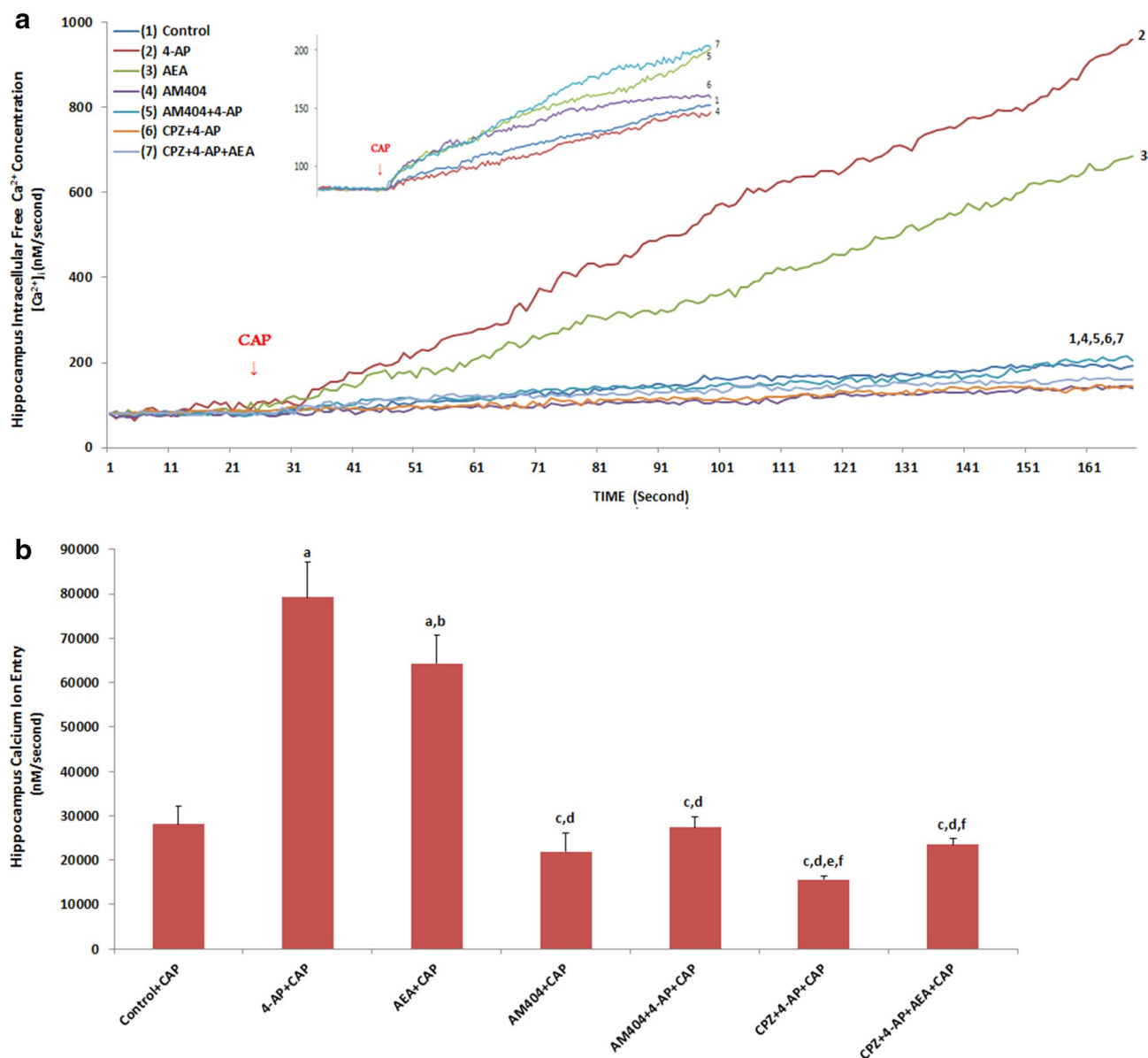


Fig. 1 Effects of AM404 and capsazepine (CPZ) treatments on $[\text{Ca}^{2+}]_i$ concentration (**a**) and entry (**b**) through inhibitions of anandamide transport and TRPV1 channel in the in vitro 4-AP seizure model-induced hippocampal neurons (mean $\pm$ SD and $n=6$). The hippocampal neurons were dissected from in vivo treated animals and they were further treated in vitro afterward with capsaicin (10 μM

and CAP) and anandamide (10 μM) although they were inhibited by CPZ (0.1 mM) and AM404 (10 μM) (^a $p<0.001$ versus control +CAP group. ^b $p<0.05$ and ^c $p<0.001$ versus 4-AP+CAP group. ^d $p<0.001$ versus AEA+CAP group. ^e $p<0.05$ versus AM404+CAP. ^f $p<0.05$ versus AM404+4-AP+CAP group)

(Fig. 1a). The mean gating time of TRPV1 in the cell was 6.03 ± 3.74 min. Induction seizure was checked by excessive Ca^{2+} entry (Fig. 2b) and induction of epileptic Ca^{2+} spikes (the data are not shown) and we observed significant current density of TRPV1 in the DBTRG cells ($p<0.001$). Then, we tested the effects of CAP and AEA on the TRPV1 current densities in AM404 + 4-AP-incubated DBTRG cells and the current densities were significantly ($p<0.001$) lower in the AM404 + 4-AP + CAP and AM404 + 4-AP + CAP + CPZ

groups than in the 4-AP+CAP group (Fig. 2c, f). We tested the increase of AEA through FAAH inhibition (via URB597 incubation) instead of inhibition of AEA transport via AM404. TRPV1 is also activated by AEA [19–21]. Because URB597 is a FAAH inhibitor chemical and we would expect further increase of 4-AP-induced TRPV1 activation through the increase of cytosolic AEA in the neuronal cell line as result of the URB597-direct effect. However, TRPV1 activity and 4-AP-induced Ca^{2+} entry were totally

blocked by URB597 and there was no response to the 4-AP-induced seizure or CAP stimulation treatments in the cells (Fig. 2d–f). The current densities of 4-AP + URB597 + CAP and URB597 + CAP groups were markedly ($p < 0.001$) lower in the 4-AP + CAP group. Therefore, we observed TRPV1 blocker and antiepileptic activity effects of URB597 in the DBTRG cell line.

Results of apoptosis and MTT levels in hippocampal neurons of rats with 4-AP-induced seizure

Figure 3 shows the effects of AEA, AM404, and CPZ on 4-AP-induced apoptosis and cell viability (MTT) levels through inhibits of AEA transport and TRPV1 channel activation in the hippocampal neurons. The apoptosis levels were significantly ($p < 0.001$) higher in the 4-AP + CAP and AEA + CAP groups than in the controls although the MTT levels were markedly ($p < 0.001$) lower in the 4-AP and AEA groups than in the controls. The 4-AP-induced apoptosis levels significantly decreased in the AEA + CAP ($p < 0.05$), AM404 + CAP ($p < 0.001$), AM404 + 4-AP + CAP ($p < 0.001$), CPZ + 4-AP + CAP ($p < 0.001$), and CPZ + 4-AP + AEA + CAP ($p < 0.001$) groups resulting from the AM404 and CPZ treatments although MTT levels were markedly ($p < 0.05$ and $p < 0.001$) increased in the groups with the AM404 and CPZ treatments.

Results of caspase 3 and 9 activities in hippocampal neurons of rats with 4-AP-induced seizure

Caspase 3 and 9 activities are inhibited in the hippocampal neurons by AM404 and CPZ (Fig. 4). The caspase 3 and caspase 9 activities were significantly ($p < 0.05$) higher in the 4-AP + CAP and AEA + CAP groups than in the controls. The 4-AP-induced caspase 3 and caspase 9 activities significantly decreased in the AEA + CAP ($p < 0.05$), AM404 + CAP ($p < 0.05$), AM404 + 4-AP + CAP ($p < 0.05$), CPZ + 4-AP + CAP ($p < 0.001$), and CPZ + 4-AP + AEA + CAP ($p < 0.001$) groups through the AM404 and CPZ treatments. In addition, the caspase activities were further decreased by CPZ treatments and their activities were markedly ($p < 0.001$) lower in the CPZ + 4-AP + CAP and CPZ + 4-AP + AEA + CAP groups than in the AM404 + CAP and AM404 + 4-AP + CAP groups.

Results of mitochondrial membrane depolarization (JC-1) and intracellular ROS production in hippocampal neurons of rats with 4-AP-induced seizure

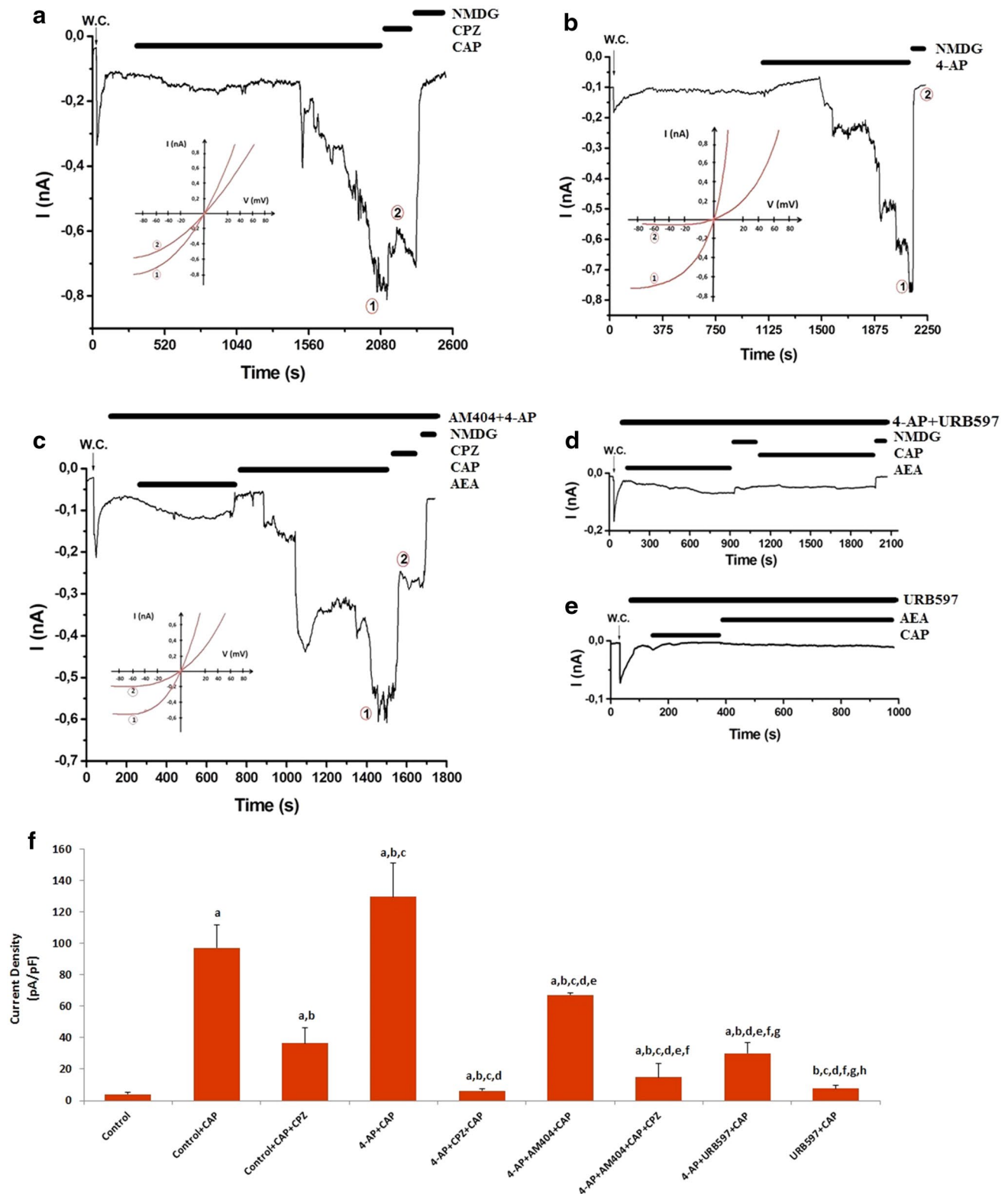
We also investigated the protective effects of CPZ and AM404 on JC-1 and ROS levels in the hippocampal

neurons (Fig. 5). The JC-1 and ROS levels were significantly ($p < 0.05$) higher in the 4-AP + CAP and AEA + CAP groups than in the controls. However, the JC-1 and ROS of hippocampal neurons were significantly ($p < 0.05$) lower in the AEA + CAP ($p < 0.05$), AM404 + CAP ($p < 0.05$), AM404 + 4-AP + CAP ($p < 0.05$), CPZ + 4-AP + CAP ($p < 0.001$), and CPZ + 4-AP + AEA + CAP ($p < 0.001$) groups. The JC-1 and ROS levels were also further decreased by CPZ treatments and their levels were markedly ($p < 0.05$) lower in the CPZ + 4-AP + CAP and CPZ + 4-AP + AEA + CAP groups than in the AM404 + CAP and AM404 + 4-AP + CAP groups.

Discussion

There are functional interactions between TRPV1 and AEA in the etiology of neuronal diseases such as anxiety, fear, and panic responses [21]. The interaction may occur in epileptic hippocampal neurons. In addition, one of the main causes of epilepsy is excessive production of oxidative stress with an overload of Ca^{2+} entry through increased seizure and mitochondrial membrane depolarization, along with TRPV1 channel activity. Our results confirm previous data [37] indicating that stimulation of AEA transport and TRPV1 channel via AEA- and CAP-induced oxidative toxicity and overload of Ca^{2+} entry in the in vitro 4-AP seizure model. However, AEA and CAP did not activate TRPV1 in the URB597-treated neurons. Hence, we observed TRPV1 blocker effects of URB597 in the DBTRG cells. However, synergistic inhibition of TRPV1 channel via CPZ, inhibition of AEA transport via AM404, and inhibition FAAH synthase via URB597 prevented the CAP- and AEA-induced $[\text{Ca}^{2+}]_i$ increase. To the best of our knowledge, there is no report of AM404, CPZ, and URB597 on apoptosis and Ca^{2+} entry through TRPV1 channels in hippocampal neurons of epileptic rats. Therefore, the results of the study clearly indicated that Ca^{2+} accumulation through activation of TRPV1 channel and AEA transport may play a regulator role in the diseases of hippocampal and DBTRG neurons.

The evidence for TRPV1 channel involvement in epileptic seizures has been indicated in a number of recent studies [4, 29]. Activation of TRPV1 channels caused overload of Ca^{2+} entry into hippocampal and dentate gyrus granule neurons and subsequent increase in action potential generation which has an important effect on induction of seizure in the neurons [4]. Recently, Umathe and Manna [22] reported in rats that intracerebroventricular administration of CAP and CPZ before seizure induction via PTZ exhibited antiepileptic action. More recently, we observed modulator and antiepileptic action of CPZ and IRTX in PTZ-induced epileptic rats [6, 23]. In the current study, CAP- and AEA-induced intracellular Ca^{2+} accumulation,



apoptosis, and oxidative stress levels were decreased in 4-AP-induced epileptic hippocampal neurons by CPZ and AM404. Hence, our results confirmed the results of other recent studies [4, 6, 22, 23].

The cannabinoid CB1 receptor-mediated effects of cannabinoids are decreased by AM404 because AEA transport through cell membranes is blocked by AM404 [15]. Apart from the effects on the endocannabinoid system, AM404

Fig. 2 Effects of anandamide (AM404 and 10 μ M for 20-min incubation), TRPV1 channel blocker (capsazepine, CPZ), and FAAH inhibitor (URB597 and 1 μ M for 60 min) treatments on TRPV1 current density in the in vitro 4-AP seizure model of DBTRG cells (mean $\pm$ SD and $n=3$). The TRPV1 currents in the DBTRG neurons were stimulated by extracellular capsaicin (CAP and 10 μ M in patch chamber) but they were blocked by extracellular CPZ (0.1 mM) in the patch chamber. Holding potential was -60 mV. Whole cell was abbreviated as W.C. **a** Control+CapN group (without AM404 and URB597). **b** 4-AP group (without AM404 and URB597). **c** AMA404+4-AP group. The neurons were incubated with AM404 (1 μ M for 20 min). Then they were incubated with 0.1 mM 4-AP before stimulation via capsaicin (10 μ M) and AEA (10 μ M). **d** 4-AP+URB597 group: The neurons were incubated with a FAAH inhibitor (URB597 and 1 μ M for 60 min). Then they were incubated with 0.1 mM 4-AP for 60 min before stimulation via capsaicin (10 μ M) and AEA (10 μ M). **e** URB597 group: The neurons were incubated with a FAAH inhibitor (URB597 and 1 μ M for 60 min) before stimulation via CAP (10 μ M) and AEA (10 μ M). **f** TRPV1 channel current densities in the DBTRG cells ($^a p \leq 0.001$ versus control. $^b p \leq 0.001$ versus control+CAP group. $^c p \leq 0.001$ versus control+CAP+CPZ group. $^d p \leq 0.001$ versus 4-AP+CAP group. $^e p \leq 0.001$ versus 4-AP+CAP+CPZ group. $^f p \leq 0.001$ versus 4-AP+AM404+CAP group. $^g p \leq 0.001$ versus AM404+4-AP+CAP+CPZ. $^h p \leq 0.001$ versus 4-AP+URB597+CAP)

activates calcium channels although the subject is somewhat controversial. For instance, a recent study demonstrated that depolarization-induced $^{45}\text{Ca}^{2+}$ currents induced by L-type VGCC are inhibited in tubule membranes of rabbit skeletal muscles by AEA although the currents were inhibited in the membranes by in vitro AM404 incubation [26]. Another study demonstrated that AM404 increases glutamate release through activation of TRPV1 receptors in rat spinal cord slices [26]. A study with limited results indicated TRPV1 activation in *Xenopus* oocytes by AM404 treatment [24]. AM404 also causes concentration-dependent relaxations in phenylephrine-induced contraction of rat hepatic artery and the relaxation effects of AM404 are blocked by CPZ [24]. To our knowledge, there is no report on interactions of TRPV1 and AM404 in neurons. In the current study, we observed modulator role of AM404 and CPZ on Ca^{2+} entry in the epileptic hippocampal neurons. In agreement with our findings, voltage-gated sodium channel [35] and T-type VGCC [38] were directly regulated by AM404. Interestingly, inhibition by AM404 of T-type VGCC was observed and the effects of AEA were blocked in the presence of AM404, suggesting that AM404 and AEA share similar sites for their actions on these channels [38]. A role for TRPV1 activations in the pharmacological actions of AM404 was supported by the data.

Reports on interaction of endocannabinoids and TRPV1 channels in epilepsy are conflicting. A recent study has demonstrated that CB1 receptor activation by endocannabinoids such as AEA or 2-arachidonoylglycerol induced neuroprotection in the hippocampus [39]. Inhibition of seizures in

epileptic models was reported for endocannabinoids [39]. In contrast, the results of a different study indicated that facilitation of epileptogenesis was reduced by decreased seizure threshold [40]. Another study reported increased TRPV1 expression in the hippocampus of patients with temporal lobe epilepsy [41]. An increase in hippocampal CB1 receptor expression and seizure rate in mice was recently reported although TRPV1 channel expression did not correlate with the repetitive seizures [5]. von Rüden et al. [5] suggested also that TRPV1 channel activity does not have a role in the etiology of epilepsy although the endovanilloid system may be strongly involved in ictogenesis or seizure induction.

It is well known that $[\text{Ca}^{2+}]_i$ accumulation has an important role on cell survival, but overload of the $[\text{Ca}^{2+}]_i$ accumulation can also induce apoptosis in neuronal death [8]. The mitochondria also have a Ca^{2+} buffering capacity by sequestering excess Ca^{2+} from the cytosol [33]. If mitochondrial membrane depolarization occurs in the hippocampus, it activates excessive ROS and RNS productions and apoptotic pathways, such as caspase 3 and caspase 9 [42]. CB1 receptor activation by AEA is important for cell viability, although inhibition of such signals has been reported to inhibit neuronal maintenance and to increase excitotoxic vulnerability [43, 44]. In addition, inhibition of the receptor induced apoptotic effect in neurons [43]. Our results indicate that the blockade of both Ca^{2+} uptake into mitochondria with AEA (AM404) and TRPV1 (CPZ) antagonists as well as increases in $[\text{Ca}^{2+}]_i$ concentration and cytosolic AEA were able to decrease mitochondrial membrane depolarization, excessive ROS production, and apoptosis mediated by AEA and CAP. Similarly, Soliman et al. [45] recently reported that apoptosis, caspase activity, and oxidative stress levels were increased by AEA incubation in a non-melanoma skin cancer line although selective inhibition of CB1 and TRPV1 did not inhibit AEA-induced oxidative stress.

AEA is hydrolyzed in cytosol of cell and neurons by FAAH [19]. URB597 is a selective inhibitor of FAAH enzyme, and it can magnify and prolong the duration of action of cytosolic produced AEA [18]. However, the presence of AEA-independent URB597-induced effects in glioblastoma cells was reported [18]. For example, TRPV1 was described as potential target of the FAAH-controlled mediators and URB597 dose dependently enhanced 2-arachidonoylglycerol and AEA level in rats. In addition, its low doses (0.5–2.5 nmol/rat) induced suppression on thermal nociception through inhibition of TRPV1 [46]. The hyperalgesic effect of URB597 at the low doses was not antagonized in rats by the CPZ injection [46]. Involvement of TRPV1 and FAAH inhibition with tonic endocannabinoid 2-arachidonoylglycerol mediated endocannabinoid signaling and GABA secretion was also reported in TRPV1 knockout mice [47]. In the current study, there was no TRPV1 activation in the seizure-induced DBTRG cells by FAAH inhibition

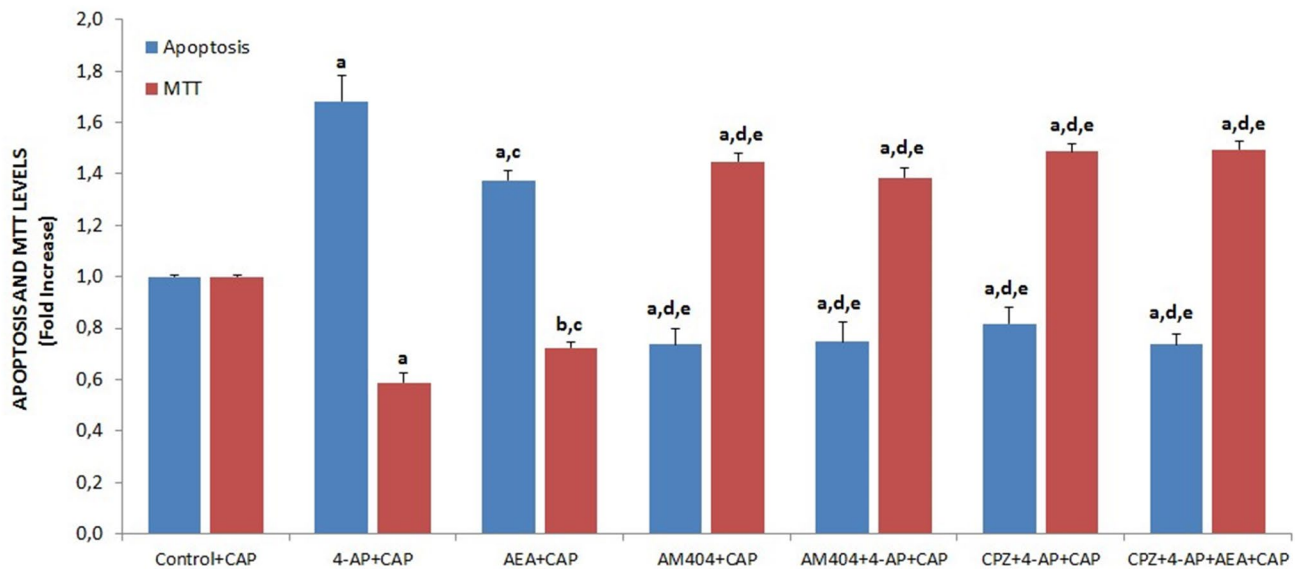


Fig. 3 Effects of AM404 and capsazepine (CPZ) treatments on apoptosis and MTT levels in the in vitro 4-AP seizure model-induced hippocampal neurons ($n=6$ and mean $\pm$ SD). Values are expressed as

fold-increase (^a $p < 0.001$ and ^b $p < 0.05$ versus control + CAP group. ^c $p < 0.05$ and ^d $p < 0.001$ versus 4-AP + CAP group. ^e $p < 0.001$ versus AEA + CAP group)

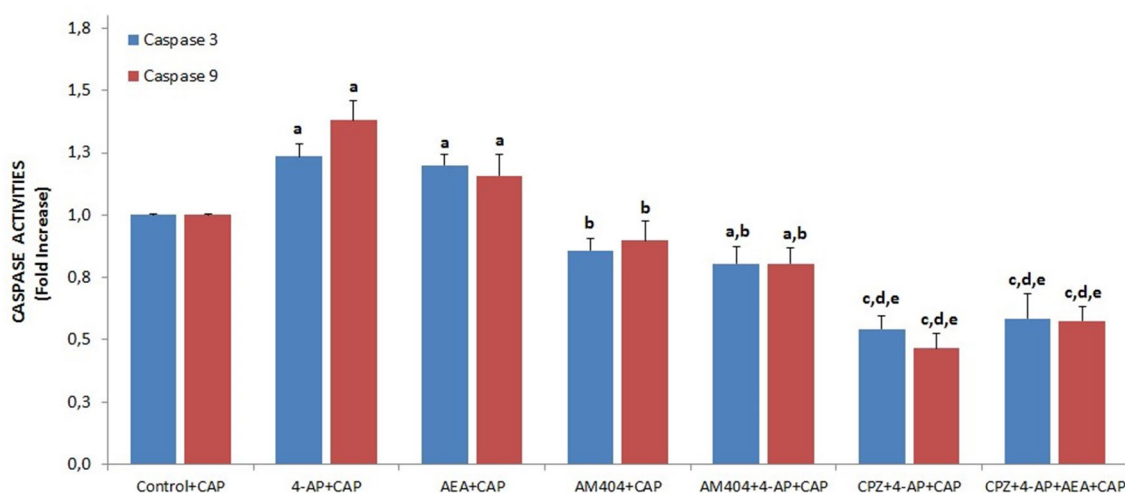


Fig. 4 Effects of AM404 and capsazepine (CPZ) treatments on caspase 3 and 9 activities in the in vitro 4-AP seizure model-induced hippocampal neurons ($n=6$ and mean $\pm$ SD). Values are expressed as

fold-increase (^a $p < 0.05$ and ^b $p < 0.001$ versus control + CAP group. ^c $p < 0.05$ and ^d $p < 0.001$ versus 4-AP + CAP and AEA + CAP groups. ^e $p < 0.001$ versus AM404 + CAP and AM404 + 4-AP + CAP groups)

through URB597 treatment. Therefore, we observed TRPV1 blocker and anti-seizure roles of URB597 in the cells. Therefore, the seizure is inhibited by inhibition of TRPV1 through the URB597 treatment. Similar to our results, antiepileptic effects of URB597 were reported in maximal dentate activation acute kindling model of the rats [48]. To our knowledge, there is no report on TRPV1 and FAAH inhibition in seizure; however, there are conflicting results on URB597 and TRPV1 activation in neuronal diseases of experimental animals. For example, anxiolytic-like effect of URB597 through

TRPV1 activation in the CA1 region of rat was recently reported [49]. Contrary to the report, anti-anxiolytic effect of URB597 and TRPV1 inhibition (via CPZ treatment) was reported in rat hippocampus [50]. It was reported that inhibition of FAAH via AM6701 treatment induced antiepileptic effects in kainic acid exposure-induced epilepsy in cultured hippocampal slices of rat pups [44].

In conclusion, the results of the current study indicate a synergistic protective effect of AEA transport blocker and TRPV1 channel antagonist but not FAAH inhibitor

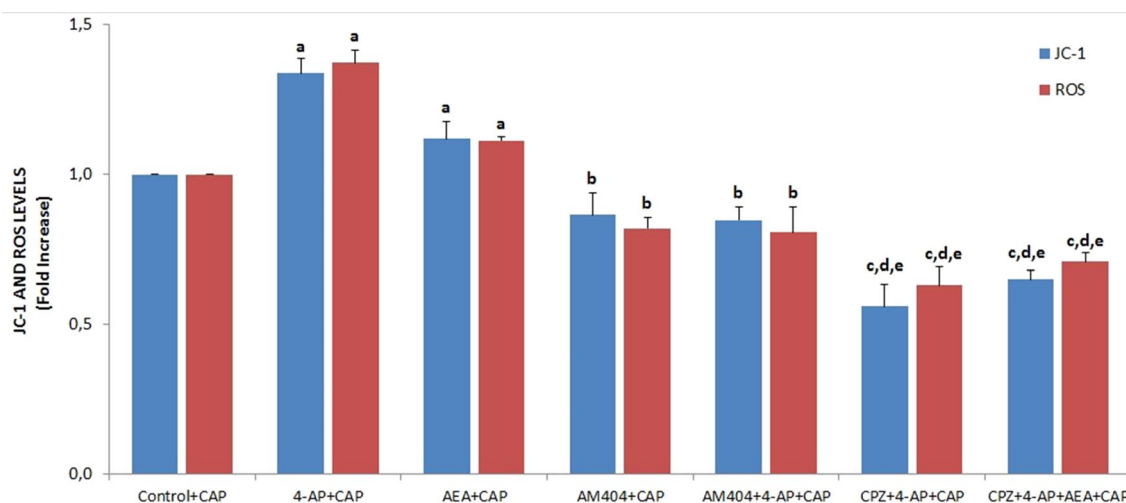


Fig. 5 Effects of AM404 and capsazepine (CPZ) treatments on mitochondrial membrane depolarization (JC-1) and intracellular ROS production in the in vitro 4-AP seizure model-induced hippocampal neurons ($n=6$ and mean $\pm$ SD). Values are expressed as fold-increase

(^a $p < 0.05$ and ^b $p < 0.001$ versus control + CAP group. ^c $p < 0.05$ and ^d $p < 0.001$ versus 4-AP + CAP and AEA + CAP groups. ^e $p < 0.05$ versus AM404 + CAP and AM404 + 4-AP + CAP groups)

on Ca^{2+} entry, oxidative stress, and apoptosis in an acute 4-AP-induced seizure model. However, a modulator role of TRPV1 channel inhibition through CPZ treatment was more important in blocking AEA transport. In addition, we observed TRPV1 blocker effects of the FAAH inhibitor (URB597) in the DBTRG cells. As Ca^{2+} entry and oxidative stress of hippocampus increase because of their elevated seizure metabolism, inhibition of TRPV1 activity and AEA transport may be useful in supporting anti-seizure activity of hippocampus. The current results represent a novel pharmacological approach toward the development of new drugs for the treatment of seizures and related syndromes resulting from overload Ca^{2+} entry and oxidative stress in the neurons.

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Author contribution All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: MN formulated the hypothesis and was responsible for writing the report. BÇ was responsible for the animal experiments. ANT and EB performed the Ca^{2+} analyses, apoptosis, and mitochondrial depolarization analyses. MN was responsible from patch-clamp analyses.

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Compliance with Ethical Standards

Conflict of interest The authors report no declarations of interest.

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