

Cellular/Molecular

Capsazepine Protects against Neuronal Injury Caused by Oxygen Glucose Deprivation by Inhibiting I_h

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Cell death mechanisms frequently involve the influx of extracellular calcium through voltage- and ligand-gated ion channels, e.g., the NMDA receptor (Greene, 1999). The vanilloid receptor (VR_1) is present in regions of the brain (Mezey et al., 2000) that are highly susceptible to neurodegenerative insults, suggesting that this ion channel might contribute to the cellular processes involved in neuronal death. We tested the effects of VR_1 ligands in the oxygen glucose deprivation (OGD) model of cell death in organotypic hippocampal slice cultures. The VR_1 agonist capsaicin at concentrations that are selective for VR_1 did not affect cell viability per se or the extent of neurodegeneration induced by the OGD insult. In contrast, the VR_1 antagonist capsazepine (0.1–10 μM) significantly reduced the amount of OGD-induced cell death. However, capsazepine was still neuroprotective in slices prepared from VR_1 knock-out mice, which exhibited the same degree of neurodegeneration to that observed in slices prepared from wild-type mice, excluding the possibility that it afforded neuroprotection through inhibition of VR_1 . Instead, capsazepine inhibited the hyperpolarization-activated nonspecific cation channel generated current I_h in a concentration range similar to that which was neuroprotective. Furthermore, the specific I_h blocker ZD-7288 was also neuroprotective, mirroring the effects of capsazepine, in that it was effective at preventing cell death when applied either during or after the OGD insult. These results demonstrate that capsazepine affords neuroprotection through inhibition of I_h rather than inhibition of VR_1 .

Key words: capsazepine; vanilloid; organotypic slice; neuroprotection; oxygen/glucose deprivation; HCN channels; I_h

Introduction

The cloning of the vanilloid receptor (VR_1), concomitant development of VR_1 -selective ligands (i.e., selective agonists, antagonists, radioligands, and monoclonal antibodies), and generation of mice genetically deficient for VR_1 have provided a major impetus toward the understanding of the physiological functions of this Ca^{2+} -permeable, nonselective cation channel. In particular, these approaches have been invaluable in demonstrating that VR_1 protein is heavily expressed in sensory neurons and that this channel plays an important role in sensory nociceptive signaling (Caterina and Julius, 2001).

However, the more widespread distribution of both VR_1 mRNA and protein in areas such as the hippocampus, striatum, hypothalamus, and cerebellum in both human and rat brain has suggested additional functional roles for this channel (Sasamura and Kuraishi, 1999; Hayes et al., 2000; Mezey et al., 2000) in physiological processes such as satiety, cognition, and motor control (Szallasi et al., 1995). Such a concept is supported by the discovery that a number of lipid molecules [e.g., anandamide and sn-2 arachidonylglycerol (Smart and Jerman, 2000)], which are present in the CNS and which can be upregulated or downregulated in line with the level of synaptic activity (Stella et al., 1997),

activate VR_1 . That this channel is permeable to Ca^{2+} also raises the possibility that it may be involved in neurodegenerative disease states. In this respect, both capsaicin and resiniferatoxin elevate intracellular free calcium and cause nuclear membrane disruption and death in cells that express VR_1 (Olah et al., 2001; Grant et al., 2002). Furthermore, alterations in environmental cues such as acid pH and heat that modulate VR_1 , and occur in response to damaging insults to the brain, may exacerbate any neurodegenerative role that this receptor might play. This is particularly pertinent to cerebral ischemia in which extracellular acidification combined with excitatory amino acid release results in increased anandamide synthesis (Hansen et al., 1995) to levels that will activate VR_1 and lead to proapoptotic injury as described in a human neuroblastoma cell line (Maccarrone et al., 2000a,b). Given these observations, we were interested to examine whether or not VR_1 contributes to neuronal cell death in intact neuronal circuits. The model system we chose to study was oxygen glucose deprivation (OGD)-induced cell death in organotypic hippocampal slices.

Materials and Methods

All procedures using animal tissues were performed using standard approved home office protocols.

Autoradiographic binding studies with [^3H]resiniferatoxin. Wild-type $+/+$ and VR_1 knock-out mice $-/-$ [postnatal day 21 (P21)] were killed by decapitation. The brains were rapidly removed, frozen, stored at -80°C , and subsequently cut into 20 μm frozen coronal brain sections.

Received July 2, 2003; revised July 2, 2003; accepted Sept. 10, 2003.

We acknowledge D. E. Owen and S. Vesce for early work that initiated these studies.

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The sections were thaw mounted onto gelatin-coated slides and stored at -80°C until time of assay.

After equilibration at room temperature, the sections were incubated in assay buffer containing the following (in mM): 5 KCl, 5.8 NaCl, 0.75 CaCl_2 , 2 MgCl_2 , 320 sucrose, and 10 HEPES, pH 7.4, containing 1 [^3H] resiniferatoxin ([^3H]RTX) (specific activity, 30–60 Ci/mmol; NEN, Boston, MA) for 60 min. Nonspecific binding was defined by the addition of 1 μM unlabelled resiniferatoxin.

After incubation, the sections were washed three times for 10 min in the assay buffer at a temperature of 4°C , briefly dipped into distilled water at 4°C to remove buffer salts, and allowed to dry in a stream of cool air. The sections were then apposed to ^3H -Hyperfilm for 5 weeks at 4°C . Adjacent sections to those used for autoradiography were stained with cresyl fast violet to permit anatomical identification of brain regions. After exposure, the sections were dipped in LM-1 nuclear emulsion to permit cellular localization of the [^3H]resiniferatoxin binding sites. After 15 weeks of exposure to emulsion, the sections were developed and stained with cresyl fast violet. Images were captured from the autoradiographic film using MCID image analysis software (Interfocus, Cambridge, UK).

Organotypic hippocampal slice culture preparation. Organotypic hippocampal slice cultures were prepared using the method of Stoppini et al. (1991). Seven- to 8-d-old Sprague Dawley rat pups were killed by cervical dislocation and decapitated, and the hippocampi were dissected out. Using a McIlwain tissue chopper, 400- μm -thick hippocampal slices were cut and placed into ice-cold growth medium consisting of 50% minimum essential medium (MEM), 25% HBSS, 25% heat-inactivated horse serum, 5 mg/ml glucose, and 1 mM glutamine. Slices with complete dentate and CA3 and CA1 areas were placed onto semiporous Millicell-CM inserts (three per membrane) and transferred to six-well plates containing 1.2 ml of growth medium. Cultures were maintained in 95% air–5% CO_2 at 37°C . Medium was changed every 4 d, and experiments were performed after 12–14 d *in vitro*. For mouse cultures, we used the same protocol as for the rat slice culture preparation, except that P6 pups were used and experiments were performed after 7–9 d *in vitro*. The VR_1 knock-out mice were prepared as described previously (Davis et al., 2000).

Oxygen glucose deprivation. For OGD experiments, the culture inserts were transferred into serum-free medium (SF) with or without compound as appropriate for 1 hr before the insult. They were then placed in deoxygenated glucose-free medium and sealed under 95% N_2 –5% CO_2 in an anaerobic chamber equilibrated to 37°C and 100% humidity (Ray et al., 2000). After 40–45 min of OGD (mouse organotypics, 35 min), cultures were removed from the chamber, and the inserts were returned to serum-free medium saturated with 95% air–5% CO_2 containing 6 $\mu\text{l/ml}$ propidium iodide (PI). Compound remained present throughout the duration of the experiment until analysis 23 hr later.

Statistical analysis. OGD-induced cell death was assayed directly in unfixed cultures using a $5\times$ lens under an inverted microscope (Zeiss, Oberkochen, Germany) equipped for fluorescence detection. Matching PI fluorescence and transmitted light images of each whole slice were recorded, and the amount of cell death in matched control and compound-treated cultures were analyzed using NIH Image software (version 1.62). The area of the cell body layer in the hippocampal CA1 area was traced from the bright-field image and superimposed over the PI image. Results were then expressed as the percentage (mean \pm SEM) of the total CA1 area expressing PI fluorescence. Statistical analysis was determined by ANOVA.

Drug application. Stock solutions of compounds were made up in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) at 10 mM. The final concentration of DMSO for any drug treatment was 0.1%, and control groups included 0.1% DMSO in all experiments. Before OGD, cultures were placed in SF containing 75% MEM, 25% HBSS, 5 mg/ml glucose, and 1 mM glutamine for 1 hr. Compound concentrations were made up from stock solutions as appropriate.

Electrophysiological recordings. Synaptic activity was recorded using either whole-cell patch-clamp or extracellular single-electrode or multi-electrode array (MEA) recording approaches. In all recording configurations, slices were bathed in warmed (32 – 37°C), perfusing (1 – 4 ml/min)

artificial CSF (aCSF) containing the following (in mM): 124 NaCl, 3 KCl, 26 NaHCO_3 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 1 MgSO_4 , and 10 D-glucose (bubbled with 95% O_2 –5% CO_2).

Whole-cell patch-clamp recordings were made under visual guidance using an Olympus Optical (Tokyo, Japan) BX50WI upright microscope. Microelectrodes (5–8 $\text{M}\Omega$) connected to an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) were filled with 135 mM K-gluconate, 10 mM MgCl_2 , 10 mM HEPES, 2 mM Mg ATP, 1 mM EGTA, 0.3 mM Na GTP, 0.1 mM CaCl_2 , pH 7.2, 290–295 mOsm. Series resistances were compensated at 80% with a lag of 8 μsec . Cell viability was assessed throughout the course of experiments via measurement of cell input resistance. Patch-clamp data were filtered at 5 kHz, digitized at 20 kHz, and stored on a personal computer hard disk drive for offline analysis using Minianalysis software and Clampfit 8.2 (Axon Instruments).

Extracellular recordings were made from individual hippocampal subfields using aCSF-filled glass microelectrodes (1–2 $\text{M}\Omega$). For evoked responses, concentric bipolar stimulating electrodes were placed to stimulate the Schaffer collaterals at the CA3 to CA1 border, and glass recording electrodes filled with aCSF were placed in the center of the CA1 stratum pyramidale. Constant current pulses were applied in 100 μA increments until a maximum response was obtained.

For spontaneous extracellular activity, recordings were made simultaneously from all subfields of hippocampal slices using a 64 electrode planar MEA purchased from MultiChannel Systems (Reutlingen, Germany). A small piece of semiporous membrane bearing the hippocampal slice was cut out of the culture well using a scalpel blade. This was positioned slice down onto an MEA, which had been coated previously using 5 μl of a solution comprising 1 cm^2 cellulose nitrate filter paper dissolved in 10 ml of methanol. The final orientation of the slice with respect to the electrodes was achieved with the aid of a Zeiss Stemi SV6 microscope. The MEA recording chamber was then immediately flooded with 1 ml of aCSF, and a photographic image was taken of the electrode positions beneath the hippocampal subfields (DC300 digital camera; Leica, Nussloch, Germany). MEA extracellular recordings were made from all subfields of the slice using the procedures outlined previously (Egert et al., 1998). MEA data were sampled at 5 kHz, stored on hard disk or CD-R, and analyzed offline using Multi Channel Systems and pClamp 9 (Axon Instruments) and Neuroexplorer (Plexon Instruments) software.

Results

Effect of capsaicin on OGD-induced cell death

Recent localization studies demonstrate expression of VR_1 immunoreactivity and VR_1 mRNA in many regions of the rodent brain, including the cortex and hippocampus (Mezey et al., 2000). Because VR_1 provides a Ca^{2+} influx pathway that might be activated in pathophysiological conditions, we hypothesized that agonists and antagonists of VR_1 might have neurotoxic and neuroprotective effects, respectively, under conditions of OGD. Before being subjected to the OGD insult, organotypic hippocampal cultures were grown for 12–14 d and examined for signs of cell death. The VR_1 full agonist capsaicin (10 μM), applied for 24 hr to control cultures, produced no toxic effect, as evaluated by the absence of any change in the low level of PI staining observed in these untreated slices (data not shown). Likewise, capsaicin (10 μM) applied from 1 hr before OGD through the OGD period and up to the 24 hr post-OGD assessment time point did not affect the extent of cell death induced by the OGD insult (data not shown).

Effect of capsazepine on OGD-induced cell death

Next we tested the ability of the VR_1 antagonist capsazepine to protect against the OGD insult. Application of capsazepine from 1 hr before and throughout the OGD insult significantly reduced the magnitude of PI staining measured 24 hr after the OGD insult (Fig. 1A). This capsazepine-induced neuroprotection was concentration dependent. Thus, 100 nM capsazepine reduced CA1 cell death by $34 \pm 8\%$, whereas 10 μM produced almost complete

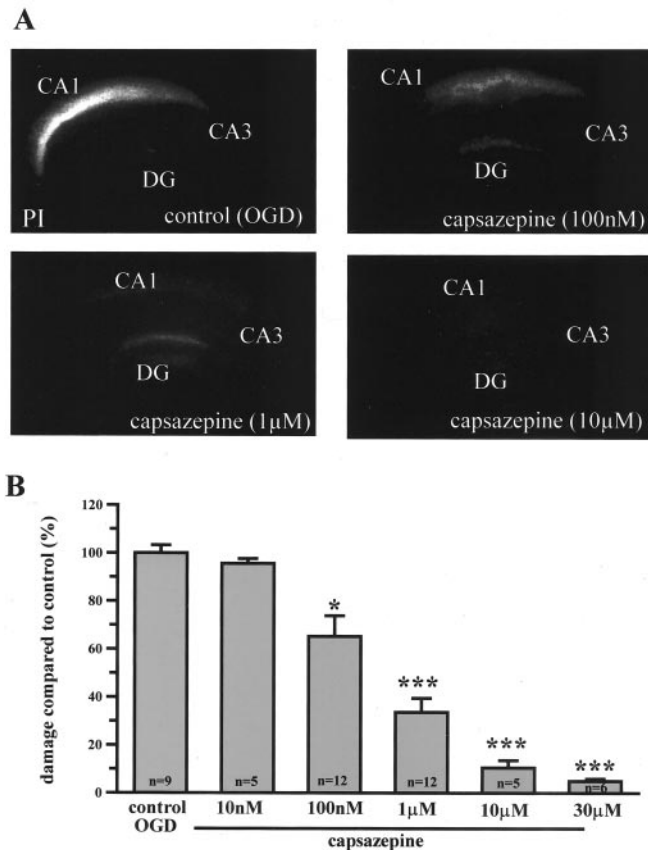


Figure 1. Concentration-dependent reduction in PI staining by capsazepine in organotypic brain slices. *A*, Propidium iodide-stained slice cultures after 45 min OGD imaged at 24 hr after insult. Control OGD, 100 nM capsazepine, 1 μ M capsazepine, 10 μ M capsazepine. *B*, Capsazepine protects hippocampal CA1 neurons from OGD-induced cell death in a concentration-dependent manner. Data are mean \pm SEM. * $p < 0.05$; *** $p < 0.0005$. DG, Dentate gyrus.

protection (Fig. 1*B*), potency in line with the activity of capsazepine at rat VR₁ (Jerman et al., 2000; McIntyre et al., 2001). Vehicle controls produced no alteration in OGD-induced PI staining.

To determine whether the neuroprotection afforded by capsazepine in slice viability experiments corresponded to functional neuroprotection, we investigated whether capsazepine also reversed the electrophysiological deficits induced by the OGD insult. As illustrated in Figure 2, the maximum amplitude of population spikes evoked in stratum pyramidale of area CA1 in response to stimulation of the Schaffer collateral commissural pathway was dramatically reduced 24 hr after the OGD insult compared with that recorded in untreated control slices. In contrast, population spikes in slices that were exposed to OGD in the presence of capsazepine were not significantly different from control ($p > 0.05$) (Fig. 2*A*). This preservation of synaptic activity was also evident in multi-electrode array recordings in which spontaneous activity present in control slices was eliminated in OGD slices but preserved in slices treated with capsazepine (Fig. 2*B*).

Post OGD treatment with capsazepine

Although capsazepine afforded clear functional protection of hippocampal synaptic activity when used as a continuous application covering the period from 1 hr before the OGD insult right up to the 23 hr post-OGD assessment point, we were interested to ascertain whether more restricted applications also proved neuroprotective. To do this, two strategies were used: (1) capsazepine

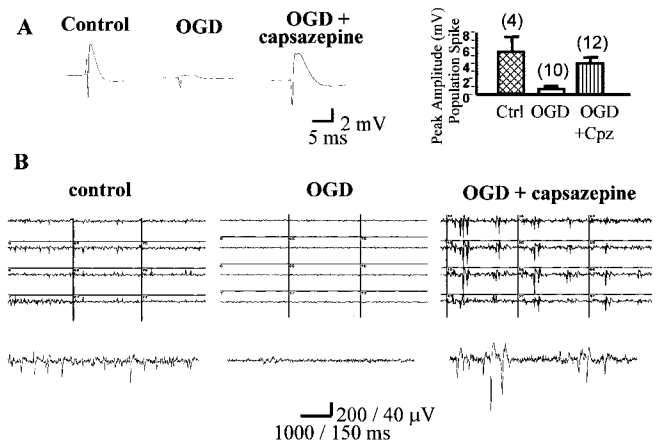


Figure 2. Functional neuroprotection with capsazepine. *A*, Maximum amplitude population spikes, recorded in stratum pyramidale of area CA1 using a 3 M NaCl filled microelectrode, from a control slice (no OGD insult), a slice that has received OGD, and an OGD plus capsazepine (10 μ M)-treated slice. The bar graph shows pooled data for the peak amplitude of the population spike evoked in these three populations of slices, with numbers of slices in parentheses. OGD-treated slices show a reduction in EPSP amplitude, and this is prevented by capsazepine. *B*, Extracellular population spike activity recorded from area CA1 of hippocampal organotypic slices. Three grids show representative 1 sec sweeps from 12 electrodes beneath area CA1. A single trace is shown below each grid on an enlarged scale. The left shows the level of spontaneous activity present in a naive control slice. OGD insult (middle) virtually abolishes all firing, whereas pretreatment with capsazepine (10 μ M; Cpz) administered 1 hr before OGD affords a clear preservation of neuronal activity recorded immediately after insult (right).

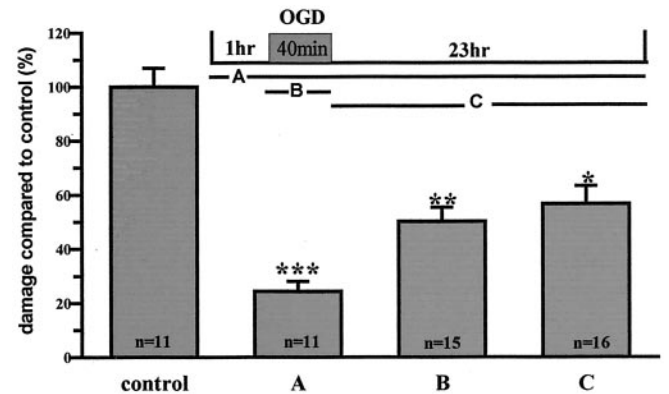


Figure 3. Capsazepine is still neuroprotective if added after OGD. Capsazepine (10 μ M) was added to the cultures at three different time points: before OGD for 1 hr, remaining throughout until analysis at 24 hr (*A*), during the OGD phase only (*B*), and after OGD only, remaining until analysis 23 hr later (*C*). Results show that there was significant protection in all cases, with the greatest effect seen with the standard pretreatment. Data are mean \pm SEM. * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$.

(10 μ M) was applied just for the period of the OGD insult, and (2) capsazepine (10 μ M) was applied starting just after the OGD insult and continuing up to the 23 hr post-OGD assessment point. In both experiments, significant neuroprotection was measured using propidium iodide staining, although the absolute magnitude of protection was slightly lower than that when a pretreatment drug regimen was used (Fig. 3). Regardless of this, these data suggest that capsazepine has protective actions both during the OGD insult and in the period after OGD.

OGD-induced cell death in VR₁ knock-out mice

Given that (1) capsazepine has been reported to inhibit a number of ion channels other than VR₁ and (2) the VR₁ agonist capsaicin

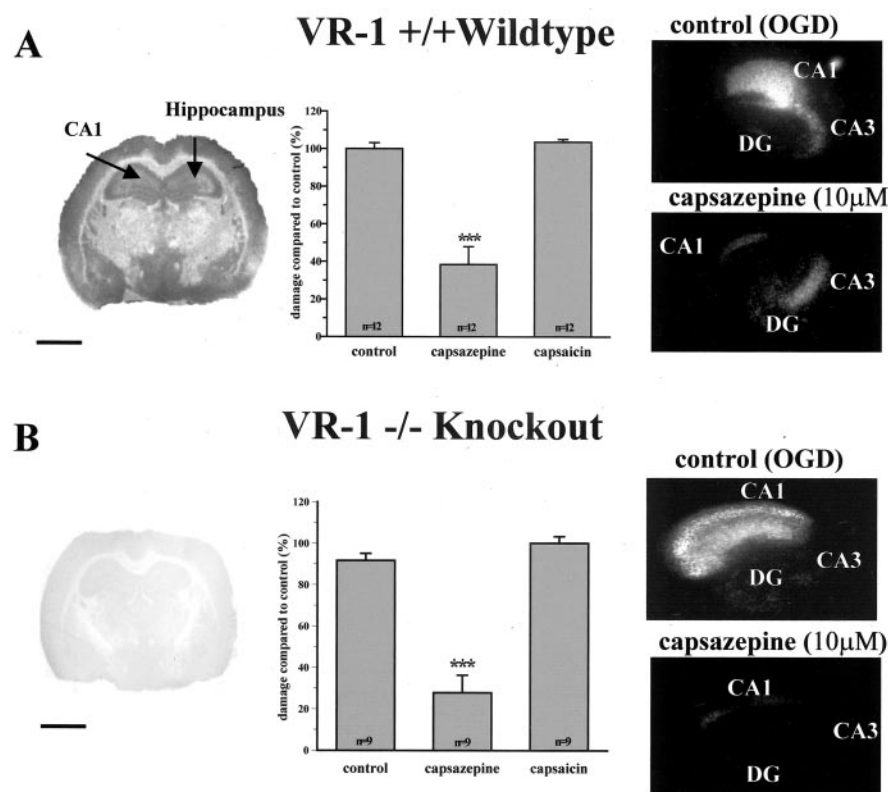


Figure 4. Capsazepine is still protective against OGD-induced cell death in VR₁ knock-out mice cultures. Distribution of specific [³H]resiniferatoxin binding in the brain of VR₁ wild-type and knock-out mice (*A*, *B*, respectively). Scale bar, 2 mm. Note the large reduction in binding seen in the knock-out brain compared with the wild type. *A*, Control damage in VR₁ +/+ mice is similar to that in VR₁ -/- mice (*B*), indicating that VR₁ is not required for OGD-induced cell death. Capsaicin has no effect in either VR₁ +/+ mice or VR₁ -/- mice, whereas capsazepine protects against CA1 damage in cultures from both VR₁ +/+ and VR₁ -/- mice. ****p* < 0.0005 (middle graphs). Images on the right show PI staining in slice cultures from either control OGD or OGD and capsazepine (10 μM). DG, Dentate gyrus.

did not in its own right cause neurotoxicity, it was possible that capsazepine was having its neuroprotective action through mechanisms other than VR₁ antagonism. To test this hypothesis, we examined whether genetic elimination of VR₁ decreased the susceptibility of VR₁ -/- (Davis et al., 2000) organotypic hippocampal slices to damage by OGD insult.

Initially, we established that VR₁ had been knocked out by comparing the pattern of [³H]RTX binding in VR₁ +/+ with that in VR₁ -/- brain. Autoradiographical analysis using this radioligand produced a strong signal compared with nonspecific binding in all neuronal layers of the hippocampus from VR₁ +/+ animals (*n* = 3) (Fig. 4*A*). Examination of the CA1 region at higher magnification revealed silver grains distributed over stratum pyramidale, oriens, and radiatum (data not shown). In contrast, sections of VR₁ -/- tissue showed levels of [³H]RTX binding that were little different from nonspecific binding (*n* = 3) (Fig. 4*B*). These results confirmed that VR₁ protein was widely distributed in all subfields of the hippocampus and that this was absent in VR₁ -/- brain.

We next proceeded to explore the functional effects of VR₁ ligands on OGD-induced cell death in VR₁ -/- organotypic hippocampal slices. To do this, we first needed to reduce the period of OGD in mouse slice cultures to 35 min to match the level of CA1 neuronal cell death to that produced by 45 min OGD in rat slice cultures. This aside, it was interesting to establish that the extent of control damage in VR₁ -/- hippocampal slices was not significantly different from that in VR₁ +/+ slices (84 ± 3 vs

77 ± 3%) (Fig. 4). These data indirectly increased the possibility that capsazepine was having its neuroprotective effects through mechanisms other than VR₁ antagonism.

To test this hypothesis, we next examined whether capsazepine prevented OGD-induced neuronal cell death in hippocampal slice cultures obtained from VR₁ -/- mice. As in rat hippocampus, capsazepine had no effect on OGD-induced cell death in either VR₁ +/+ or VR₁ -/- slices (Fig. 4*A, B*). However, 10 μM capsazepine administered from 1 hr before OGD and throughout the insult until the 23 hr post-OGD assessment time point afforded similar levels of neuroprotection in both VR₁ +/+ and VR₁ -/- slice cultures to that observed in rat hippocampal slices. Together, these results provide convincing evidence that VR₁-activated cellular processes are not involved in OGD-induced neurodegeneration in the hippocampus and suggest that the neuroprotective mechanism of capsazepine operates via an alternative process to simple blockade of VR₁.

Capsazepine neuroprotection: an action on other receptors or ion channels?

Given these data, we next examined whether any of the other pharmacological actions that have been described for capsazepine could account for its neuroprotective effects. In a first series of experiments, we examined whether capsazepine

affected both ionotropic and metabotropic glutamate receptors (mGluRs) because antagonism of these receptors has been widely reported to afford functional neuroprotection *in vitro*. As illustrated in Figure 5*A*, capsazepine had no effect on the frequency or amplitude of spontaneous AMPA or GABA_A receptor-mediated postsynaptic currents recorded in organotypic slice cultures. Furthermore, capsazepine had no effect on either bicuculline (10 μM) induced epileptiform activity (*n* = 6) (Fig. 5*B*), which is dependent on activation of both NMDA and AMPA receptors, or mGluR-dependent synchronized population bursting induced by application of the group I mGluR agonist (*RS*)-3,5-dihydroxyphenylglycine (DHPG) (20 μM) (frequency of bursting and peak amplitude of individual bursts in the absence and presence of capsazepine were 16.2 ± 2.6 and 17.4 ± 1.4 Hz and 208 ± 13 and 205 ± 16 pA, respectively; *n* = 3) (Fig. 5*C*).

In a second series of experiments, we examined whether capsazepine affected TTX-sensitive voltage-gated sodium channels in organotypic hippocampal slices because inhibition of these channels affords complete neuroprotection to OGD. In three experiments, capsazepine did not affect voltage-gated sodium currents such that the peak amplitude of the Na⁺ current in response to a 20 mV depolarizing step was 4143 ± 132 pA in control medium as opposed to 4457 ± 141 pA in the presence of capsazepine (Fig. 5*D*).

In a third series of experiments, we examined the unlikely possibility that the well documented antagonism of nicotinic acetylcholine receptors by capsazepine (Liu and Simon, 1997) ac-

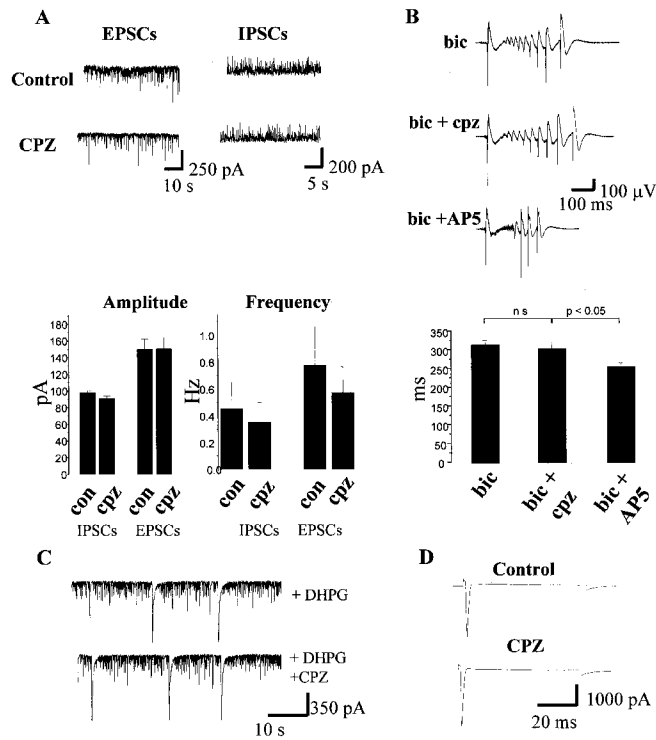


Figure 5. Capsazepine does not effect AMPA or NMDA receptor-mediated transmission and has no effect on voltage-gated sodium channels. *A*, Traces on the left are whole-cell recordings of spontaneous EPSCs sampled from a CA1 pyramidal neuron holding at -70 mV in control medium and in the presence of $10 \mu\text{M}$ capsazepine (CPZ). Traces on the right are IPSCs sampled from another CA1 pyramidal neuron under control conditions and in the presence of $10 \mu\text{M}$ capsazepine. The histograms illustrate the absence of effect of capsazepine ($10 \mu\text{M}$) on the frequency and amplitude of EPSCs and IPSCs. *B*, Traces from top to bottom are synchronized bursts recorded in bicuculline ($10 \mu\text{M}$; bic)-containing medium in the added presence of $10 \mu\text{M}$ capsazepine (cpz) and in the added presence of $50 \mu\text{M}$ D-AP-5 (AP5). The bar graph below shows pooled data for six experiments demonstrating no significant effect of capsazepine and a significant reduction in burst duration by the NMDA receptor antagonist D-AP-5. *C*, Traces are taken from the same experiment illustrating spontaneous EPSCs in *A* but represent activity in the presence of $20 \mu\text{M}$ DHPG. Note that DHPG causes a significant increase in the frequency of postsynaptic currents as well as synchronized bursting activity, neither of which were modified by capsazepine. *D*, Voltage-gated sodium channel currents induced by a step from -70 to -50 mV (duration, 100 msec) are not modified by capsazepine. These traces are averages of five sweeps each.

counted for the neuroprotective action of capsazepine. To do this, we took an alternative approach and examined whether the selective nicotinic acetylcholine receptor (nAChR) antagonists α -methylcaconitine ($10 \mu\text{M}$) and dihydro- β -erythroidine ($10 \mu\text{M}$) affected OGD-induced cell death in rat organotypic hippocampal slices. In every experiment performed, neither antagonist affected the magnitude of neuronal cell death induced by the OGD insult (Fig. 6)

Having exhausted these possible mechanisms as the pathway by which capsazepine affords neuroprotection, we cast our net of potential mechanisms much wider by examining the possibility that capsazepine might inhibit voltage-gated channels related to VR_1 . Specifically, we chose to examine whether capsazepine inhibited the hyperpolarization-activated nonspecific cationic channel because this is highly expressed in the hippocampus and is likely to be involved in synchronization–patterning of neuronal activity that is believed to occur as a result of the OGD insult. First, we chose to examine whether capsazepine inhibited the current (I_h) that is generated in hippocampal neurons when they are hyperpolarized from -50 to -140 mV for 1 sec. In all neu-

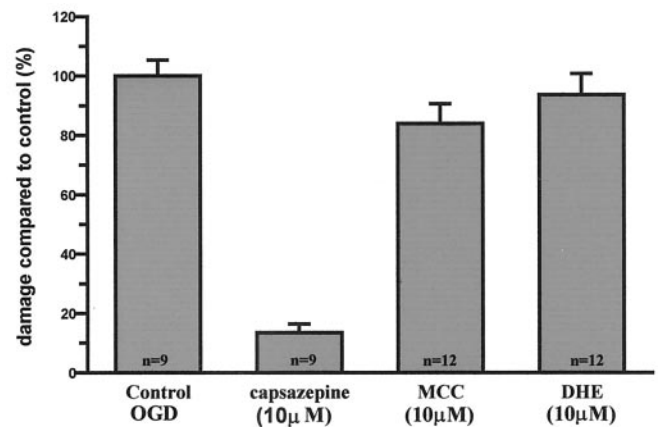


Figure 6. Nicotinic receptor antagonists are not neuroprotective against OGD-induced cell death. Effect of the broad-spectrum nAChR antagonist dihydro- β -erythroidine ($10 \mu\text{M}$; DHE) and the $\alpha 7$ nAChR-selective antagonist α -methylcaconitine ($10 \mu\text{M}$; MCC) on OGD-induced cell death in organotypic slice cultures. $n = 12$ each group. Data are mean \pm SEM and normalized to control.

rons tested, capsazepine ($10 \mu\text{M}$) produced a $42.3 \pm 2.8\%$ inhibition of I_h ($n = 3$), whereas the specific I_h inhibitor ZD-7288 at the same concentration afforded a $9.26 \pm 5.3\%$ block ($n = 3$).

Given these findings and that capsazepine produced almost complete inhibition of OGD-induced cell death at $10 \mu\text{M}$, we were interested to examine whether ZD-7288 was also able to prevent OGD-induced cell damage. Application of ZD-7288 from 1 hr before and throughout the OGD insult significantly reduced the magnitude of PI staining measured 24 hr after the OGD insult (Fig. 7). This effect was concentration dependent such that 100 nM ZD-7288 reduced CA1 cell death by $19.14 \pm 4.6\%$, whereas $1 \mu\text{M}$ produced almost complete protection, with vehicle controls producing little or no effect. To further characterize this neuroprotective effect of ZD-7288, we examined whether functional neuroprotection was obtained by investigating whether this compound also reversed the electrophysiological deficits induced by the OGD insult. As illustrated in Figure 7, ZD-7288 significantly reversed the reduction in maximum population spike amplitude that is observed 24 hr after the OGD insult in untreated control slices.

Although these results closely paralleled those of capsazepine treatment, we wanted to confirm that ZD-7288 was also capable of providing neuroprotection when it was applied after the OGD insult because this was another activity exhibited by capsazepine. To do this, ZD-7288 ($10 \mu\text{M}$) was applied starting just after the OGD insult and continuing up to the 23 hr post-OGD assessment point. As predicted, if capsazepine was affording neuroprotection through inhibition of I_h , ZD-7288, when applied after the OGD insult, prevented the increase in propidium iodide staining that was observed in control slices.

Discussion

In this study, we showed that VR_1 protein is abundantly expressed in the hippocampus and that, in a hippocampal slice culture model using both rat and mouse organotypic slices, capsaicin is not neurotoxic. Capsazepine on the other hand is a potent neuroprotective agent against OGD-induced damage. However, this neuroprotection is not via an action on VR_1 . To gain insight into the mechanism of action of capsazepine, we investigated its actions on other receptors and channels within the slice

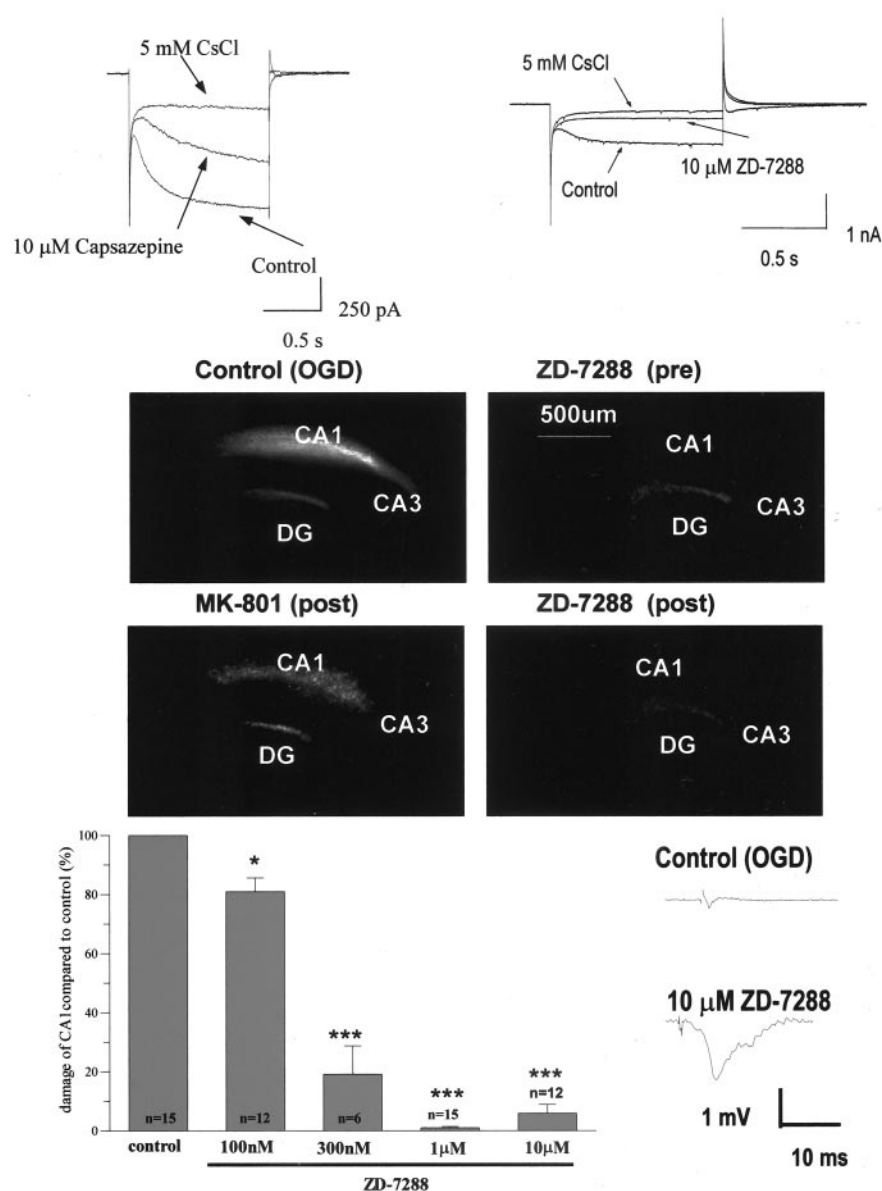


Figure 7. Capsazepine inhibits I_h , and the specific I_h blocker ZD-7288 is neuroprotective against OGD-induced cell death. *A*, Sample traces from whole-cell patch-clamp recordings of hyperpolarized activated cation current. Left shows the significant inhibitory effect of capsazepine, and the right shows near-complete block of the current by the specific I_h blocker ZD-7288. In each panel, responses in the presence of CsCl, which abolishes the current, are included as a reference. *B*, PI images of organotypic hippocampal slice cultures treated with vehicle (Control), ZD-7288 (1 μ M), or MK-801 (positive control) (10 μ M). Compound was given either 1 hr before OGD or immediately after OGD. Images were taken 24 hr after insult. *C*, Dose–response data for ZD-7288. * $p < 0.05$; *** $p < 0.0005$. Data are mean \pm SEM and normalized to control. *D*, Typical field EPSPs recorded in the CA1 region of the hippocampus. The presence of ZD-7288 substantially reversed the reduction in amplitude of responses in slices subjected to OGD.

and discuss further potential targets in which capsazepine may have an action.

VR₁ agonist effects in slice cultures

In our experiments, capsaicin showed no deleterious effects on either naive slice cultures or cultures exposed to OGD in the presence of capsaicin. In contrast, in other purely cell-based systems, agonists of VR₁ have induced apoptotic mechanisms resulting in cell death (Maccarrone et al., 2000a). This apoptotic cell death is usually linked to an increase in internal calcium levels and activation of certain proteases (Chard et al., 1995). The reason for this difference is not clear. One possibility is that, in

organotypic slices, the expression of VR₁ on individual neurons may be so low that any intracellular rise in Ca²⁺ is easily buffered, by intracellular Ca²⁺ binding proteins, to a level that is below that which is neurotoxic. Alternatively, the complex interactions between glia and neurons in organotypic cultures may conspire together to attenuate any neurotoxic effect of capsaicin by activation of endogenous neuroprotective cellular processes within the slice.

Capsazepine is neuroprotective in slice cultures

Our studies using the OGD model have shown that capsazepine is neuroprotective in that it prevents both changes in cell number and alterations in macroscopic synaptic function that normally occur in response to this insult. Taken at face value, these results could be interpreted as a neuroprotective action through antagonism of the Ca²⁺-permeable nonspecific cation channel VR₁. However, when viewed in conjunction with the observation that capsazepine is still neuroprotective in hippocampal slices derived from VR₁ knockout mice, it appears that capsazepine is acting through an alternative mechanism(s) to VR₁ antagonism to produce its neuroprotective effect. Furthermore, not only does VR₁ not appear to be an essential component in OGD neurodegeneration, its sustained activation does not result in marked changes in neuronal excitability or cellular deterioration, because 10 μ M capsaicin, which is sufficient to maximally activate VR₁ in sensory neurons (Bevan et al., 1992), had no overt effects on synaptic electrophysiology or cell viability.

Thus, if the neuroprotective action of capsazepine does not result from an interaction with VR₁, how does capsazepine afford neuroprotection? The VR₁ receptor, although clearly unique in its own right, does share many molecular and biophysical properties that are displayed by other members of the voltage-gated and ligand-gated ion channel families. It is not surprising, therefore, that capsazepine has been reported to inhibit a variety of ligand- and voltage-gated ion channels that, like VR₁, are cation permeable.

AMPA and NMDA receptors

Antagonism of NMDA receptors has been shown to be extremely effective in preventing OGD-induced neurodegeneration (Maier et al., 1995; Werth et al., 1998; Pringle et al., 2000). Likewise, blockade of AMPA receptors can afford neuroprotection (Strasser and Fischer, 1995; LoPachin et al., 2001). However, these are unlikely to be the target mechanisms that explain the neuroprotective effect of capsazepine because capsazepine did

not affect AMPA or NMDA receptor-mediated synaptic transmission. The inactivity of capsazepine as a group I mGluR antagonist is also noteworthy because both mGluR1 and mGluR5 antagonists have been shown recently to prevent excitotoxic neurodegeneration (Bruno et al., 1999, 2000).

Sodium and potassium channels

Capsazepine inhibits both voltage-gated sodium and potassium channels (Kuenzi and Dale, 1996; Su et al., 1999). However, these nonspecific actions are observed at concentrations in excess of 10 μM , which are 10- to 100-fold higher than the concentrations that afford significant levels of neuroprotection in the present study. Furthermore, we could not demonstrate any effect of capsazepine on TTX-sensitive voltage-gated sodium currents in our hippocampal organotypic slice cultures. Thus, it seems unlikely that modification of these cationic channels is a major contributing factor to the neuroprotective mechanisms invoked by capsazepine in the OGD model.

Voltage-operated calcium channels

Because the magnitude of OGD neurodegeneration is dependent on extracellular calcium, it is possible that inhibition of voltage-gated Ca^{2+} channels could potentially provide a mechanism of neuroprotection. However, although capsazepine has been reported to inhibit voltage-activated calcium channels (Docherty et al., 1997), it is unlikely that this activity is responsible for its neuroprotective action because (1) the IC_{50} for capsazepine-induced voltage-gated Ca^{2+} channel blockade is 1.4–10 μM , whereas the IC_{50} for its neuroprotective effect is 0.1–0.3 μM , and (2) studies using the high-voltage-activated calcium channel antagonists ω -conotoxin MVIIC, which blocks N type as well as other presynaptic calcium channels, nifedipine, which blocks L type channels, and SB201823-A, which is a broad-spectrum calcium channel blocker, provided little or no protection against OGD insults in organotypic hippocampal cultures (Pringle et al., 1996).

Nicotinic acetylcholine receptor

Nonglutamatergic ligand-gated ion channels have also been implicated as targets affording neuroprotection. One in particular is the nAChR (Marin et al., 1994). However, although capsazepine has been shown to be active against this ligand-gated receptor, it acts as an antagonist (Liu and Simon, 1997), and it is generally accepted that nicotinic agonists, and not antagonists, afford neuroprotection (Dajas-Bailador et al., 2000). Furthermore, in our own studies, we showed that the broad-spectrum nAChR antagonist dihydro- β -erythroidine and the $\alpha 7$ nAChR-selective antagonist α -methylaconitine suggest that antagonism of nAChRs does not protect against OGD-induced neurodegeneration.

Hyperpolarization activated cyclic nucleotide modulated channel-1 channels

An interesting and novel finding of this study, however, was that capsazepine inhibited the hyperpolarization-activated nonspecific cation channel-mediated current I_h . We believe that it is this activity that underlies the neuroprotective effects of capsazepine because (1) the concentration range over which capsazepine inhibited I_h was similar to that which afforded functional neuroprotection, and (2) the specific I_h blocker ZD-7288 exhibited a very similar profile of neuroprotection to that afforded by capsazepine in that it prevented cell death when applied either before or after the OGD insult. Recently, the specificity of ZD-7288 as a selective I_h blocker has been called into question (Chevalyere and

Castillo, 2002). However, in the current study, we are confident that its effects are related to inhibition of I_h because it was effective in a concentration range that inhibits I_h , and, equally importantly, this range is well below that reported to inhibit glutamatergic synaptic transmission. That inhibition of I_h is neuroprotective can be rationalized by the well documented observations that ZD-7288 hyperpolarizes hippocampal neurons and prevents concerted synchronized bursting of the form likely to produce neuronal damage.

Concluding remarks

Given the data presented in this study that rule out previously identified pharmacological activities of capsazepine as the site of action of its neuroprotective activity, we feel that it is justified to conclude that capsazepine mediates its neuroprotective effects, at least in part, through inhibition of I_h . That said, we cannot conclusively rule out the possibility that it may mediate part of its effects through (1) conjoint activation and/or inhibition of a number of cellular processes or (2) additional pharmacological properties yet to be discovered, e.g., block of an, as yet, undisclosed voltage-gated channel and possibly a member of the transient receptor potential channel family to which VR_1 and hyperpolarization activated cyclic nucleotide modulated channel-1 channels are closely related (Caterina et al., 1997). This aside, that inhibition of I_h provides neuroprotection is a particularly novel and interesting finding, not least because this mechanism is effectively neuroprotective when implemented either before or after the OGD insult. Most importantly, this pattern of activity is what is required to produce a useful therapeutic agent for stroke and other head trauma injuries as these patient groups are usually defined after the initial neurodegenerative insult when treatment is restricted to preventing the secondary damage that occurs after that induced by the insult itself.

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