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 (VR1) 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 VR1 ligands in the oxygen glucose deprivation (OGD) model of cell death in organotypic hippocampal slice cultures. The VR1 agonist capsacin at concentrations that are selective for VR1 did not affect cell viability per se or the extent of neurodegeneration induced by the OGD insult. In contrast, the VR1 antagonist capsazepine (0.1–10 μM) significantly reduced the amount of OGD-induced cell death. However, capsazepine was still neuroprotective in slices prepared from VR1 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 VR1. 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 VR1.

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

Introduction

The cloning of the vanilloid receptor (VR1), concomitant development of VR1-selective ligands (i.e., selective agonists, antagonists, radioligands, and monoclonal antibodies), and generation of mice genetically deficient for VR1 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 VR1 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 VR1 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 arachidonylethanolamide (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 VR1. 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 VR1 (Olah et al., 2001; Grant et al., 2002). Furthermore, alterations in environmental cues such as acid pHi and heat that modulate VR1, 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 VR1 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 VR1 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 VR1 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.
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 decapsulation, 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% mini-embryo medium 2 (MEM 2) supplemented with 45 mM CaCl2, 2.5 mM MgCl2, 2.5 mM KCl, and 10 mM HEPES, pH 7.4, containing 1 μM resi- niferatoxin ([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.

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 NaHCO3, 1.25 NaH2PO4, 2 CaCl2, 1 MgSO4, and 10 n-glucose (bubbled with 95% O2–5% CO2).

Whole-cell patch-clamp recordings were made using visual guidance using an Olympus Optical (Tokyo, Japan) BX50W1 upright microscope. Whole microelectrodes (5–9 MΩ) were connected to a listachip 2008 amplifier (Axon Instruments, Foster City, CA) and filled with 135 mM K-glucionate, 10 mM MgCl2, 10 mM HEPES, 2 mM Mg ATP, 1 mM EGTA, 0.3 mM Na GTP, 0.1 mM CaCl2, pH 7.2, 290–295 μSm. 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 3 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 MΩ). 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 simulta-neously from all subfields of hippocampal slices using a 64 electrode planar MEA purchased from MultiChannel Systems (Reutlingen, Germany). A piece of semimembrane 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 cm2 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, Nuss-loch, 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 3 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 capsazepine on OGD-induced cell death

Recent localization studies demonstrate expression of VR1 im-munoreactivity and VR1 mRNA in many regions of the rodent brain, including the cortex and hippocampus (Mezey et al., 2000). Because VR1 provides a Ca2+ influx pathway that might be activated in pathophysiological conditions, we hypothesized that agonists and antagonists of VR1 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 VR1 full agonist capsazepine (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, capsazepine (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 VR1 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 ± 8%, whereas 10 μM produced almost complete
protection (Fig. 1B), 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. 2A). 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. 2B).

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 (10 μM) was applied just for the period of the OGD insult, and (2) capsazepine (10 μM) 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...
did not in its own right cause neurotoxicity, it was possible that capsaizpine was having its neuroprotective action through mechanisms other than VR 1 antagonist. To test this hypothesis, we next examined whether capsaizpine prevented OGD-induced neuronal cell death in hippocampal slice cultures obtained from VR 1−/− mice. As in rat hippocampus, capsaizpine had no effect on OGD-induced cell death in either VR 1+/+ or VR 1−/− slices (Fig. 4A, B). However, 10 μM capsaizpine 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 VR 1+/+ and VR 1−/− slice cultures to that observed in rat hippocampal slices. Together, these results provide convincing evidence that VR 1−-activated cellular processes are not involved in OGD-induced neurodegeneration in the hippocampus and suggest that the neuroprotective mechanism of capsaizpine operates via an alternative process to simple blockade of VR 1.

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 capsaizpine could account for its neuroprotective effects. In a first series of experiments, we examined whether capsaizpine 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 5A, capsaizpine had no effect on the frequency or amplitude of spontaneous AMPA or GABA A receptor-mediated postsynaptic currents recorded in organotypic slice cultures. Furthermore, capsaizpine had no effect on either bicuculline (10 μM) induced epileptiform activity (n = 6) (Fig. 5B), 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 capsaizpine were 16.2 ± 3 Hz and 208 ± 13 pA, respectively; n = 3) (Fig. 5C).

In a second series of experiments, we examined whether capsaizpine affected TTX-sensitive voltage-gated sodium channels in organotypic hippocampal slices because inhibition of these channels affords complete neuroprotection to OGD. In three experiments, capsaizpine 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 ± 141 pA in control medium as opposed to 4457 ± 141 pA in the presence of capsazepine (Fig. 5D).

In a third series of experiments, we examined the unlikely possibility that the well documented antagonism of nicotinic acetylcholine receptors by capsaizpine (Liu and Simon, 1997) ac-
First, we chose to examine whether capsazepine inhibited the activity that is believed to occur as a result of the OGD insult. Capsazepine is likely to be involved in synchronization because this is highly expressed in the hippocampus and 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 (Ih) that is generated in hippocampal neurons when they are hyperpolarized from −50 to −140 mV for 1 sec. In all neurons tested, capsazepine (10 μM) produced a 42.3 ± 2.8% inhibition of Ih (n = 3), whereas the specific Ih inhibitor ZD-7288 at the same concentration afforded a 9.26 ± 5.3% block (n = 3).

Given these findings and that capsazepine produced almost complete inhibition of OGD-induced cell death at 10 μM, 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 ± 4.6%, whereas 1 μM 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 μM) was applied starting just after the insult because this was another activity exhibited by capsazepine. 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 μM) was applied starting just after the OGD insult because this was another activity exhibited by capsazepine. To do this, ZD-7288 (10 μM) was applied starting just after the OGD insult because this was another activity exhibited by capsazepine. To do this, ZD-7288 (10 μM) was applied starting just after the OGD insult because this was another activity exhibited by capsazepine. To do this, ZD-7288 (10 μM) was applied starting just after the OGD insult because this was another activity exhibited by capsazepine.
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 Ca$^{2+}$ is easily buffered by intracellular Ca$^{2+}$ 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 Ca$^{2+}$-permeable nonspecific cation channel VR1. However, when viewed in conjunction with the observation that capsazepine is still neuroprotective in hippocampal slices derived from VR1 knock-out 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 μM 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 induce 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 MVIIIC, 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 α7 nAChR-selective antagonist α-methylcyclooctamine 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 (Chevaleyre 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 VR1, 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.

**References**


