Activation Of A Novel Injury-Induced Calcium Permeable Channel That Plays A Key Role In Causing Extended Neuronal Depolarization and Initiating Neuronal Death in Excitotoxic Neuronal Injury

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Abbreviations: END (Extended Neuronal Depolarization), I_EIC (Excitotoxic Injury Current), VGCC (Voltage-gated Calcium Channels)

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Abstract- Protracted elevation in intracellular calcium caused by the activation of the N-methyl-D-aspartate receptor is the main cause of glutamate excitotoxic injury in stroke. However, upon excitotoxic injury, despite the presence of calcium entry antagonists, calcium unexpectedly continues to enter the neuron causing extended neuronal depolarization and culminating in neuronal death. This phenomenon is known as the calcium paradox of neuronal death in stroke and represents a major problem in developing effective therapies for the treatment of stroke. To investigate this calcium paradox and to determine the source of this unexpected calcium entry after neuronal injury, we evaluated whether glutamate excitotoxicity activates an injury induced calcium permeable channel responsible for conducting a calcium current that underlies neuronal death. We employed a combination of whole-cell and single-channel patch clamp recordings, fluorescent calcium imaging and neuronal cell-death assays in a well-characterized primary hippocampal neuronal culture model of glutamate excitotoxicity/ stroke. Here we report activation of a novel calcium permeable channel upon excitotoxic glutamate injury that carries calcium current even in the presence of calcium entry inhibitors. Blocking this injury-induced calcium permeable channel for a significant time period after the initial injury is still effective in preventing calcium entry, extended neuronal depolarization and delayed neuronal death, thus accounting for the calcium paradox. This injury induced-calcium permeable channel represents a major source for the initial calcium entry following stroke and offers a new target for extending the therapeutic window for preventing neuronal death after the initial excitotoxic (stroke) injury.
Introduction

Stroke is a leading cause of disability and death, yet it’s successful treatment is limited (Bonita et al., 2004). Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system and is required for neural development, synaptogenesis, and alterations in synaptic plasticity (Dingledine et al., 1999). In excessive quantities, glutamate is believed to cause the neuronal damage observed following stroke, epilepsy, and traumatic brain injury (Siesjo and Bengtsson, 1989; Delorenzo et al., 2005). Protracted elevations in intracellular calcium ([Ca\(^{2+}\)]\(_i\)) caused by the activation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors is the main cause of excitotoxic injury in stroke (Choi, 1995; Lipton, 1999). Excitotoxic glutamate exposure causes two major, long-lasting changes in neuronal physiology. Our laboratory and others have demonstrated that glutamate exposure results in a prolonged disturbance in neuronal Ca\(^{2+}\) homeostasis, producing protracted elevations in [Ca\(^{2+}\)]\(_i\) that persist following the removal of glutamate (Glaum et al., 1990; Michaels and Rothman, 1990; Dubinsky, 1993; Choi, 1995; Limbrick et al., 1995; Limbrick et al., 2001; Limbrick et al., 2003). In addition, excitotoxic glutamate exposure causes extended neuronal depolarization (END), a condition that lasts for several hours after glutamate removal (Sombati et al., 1991; Coulter et al., 1992; Limbrick et al., 2003). Both the protracted [Ca\(^{2+}\)]\(_i\) elevations and END follow similar time courses, require NMDA receptor activation and an increase in [Ca\(^{2+}\)]\(_i\) for induction, and correlate with excitotoxic neuronal cell death (Sombati et al., 1991; Coulter et al., 1992; Limbrick et al., 2003).

Conventional Ca\(^{2+}\) entry antagonists prevent neuronal death and END when administered before and during the injury phase of glutamate excitotoxicity (Coulter et al., 1992; Limbrick et al., 2001), but after excitotoxic insult has occurred, these Ca\(^{2+}\) entry antagonists are no longer
effective in blocking the Ca$^{2+}$ entry and reducing the elevated [Ca$^{2+}$]i (the Ca$^{2+}$ plateau). Thus, traditional Ca$^{2+}$ entry antagonists do not prevent END (Limbrick et al., 2003), block neuronal death (Ikonomidou and Turski, 2002) or improve the outcome after the excitotoxic injury in stroke (Horn and Limburg, 2000). These observations have lead to the Ca$^{2+}$ paradox of neuronal death in stroke and refers to the unexpected finding that conventional Ca$^{2+}$ entry antagonists do not prevent Ca$^{2+}$ entry or END after glutamate excitotoxicity (Lee et al., 1999; Horn and Limburg, 2001; Ikonomidou and Turski, 2002). Explaining the cause of the Ca$^{2+}$ paradox of neuronal death in stroke is one of the important problem in neuroscience research and underlies the failure of many of the previous clinical trials for potential neuroprotective agents in stroke and brain injury (Lee et al., 1999; Horn and Limburg, 2001; Ikonomidou and Turski, 2002; Wahlgren and Ahmed, 2004). Our previous attempts at elucidating the Ca$^{2+}$ paradox identified that an influx of extracellular Ca$^{2+}$ was underlying the genesis of END. However that study couldn’t identify the source or nature of this post-injury Ca$^{2+}$ entry (Limbrick et al., 2003). It is therefore important to explain this Ca$^{2+}$ paradox and understand the continued Ca$^{2+}$ entry after injury despite the use of known Ca$^{2+}$ entry inhibitors in order to develop novel and effective stroke and brain injury therapeutic agents.

In this study using a well-characterized in vitro hippocampal neuronal culture model of glutamate excitotoxicity/ stroke we investigated the cause of the Ca$^{2+}$ entry after injury that is resistant to known Ca$^{2+}$ entry inhibitors. Experiments were directed at evaluating the development of an injury induced Ca$^{2+}$ current that underlies END and cell death that is not blocked by conventional Ca$^{2+}$ entry inhibitors. Studies were also directed at demonstrating that this novel injury induced Ca$^{2+}$ current accounts for the Ca$^{2+}$ paradox, since traditional Ca$^{2+}$ entry inhibitors including blockers for L, N, P/Q, T-type Ca$^{2+}$ channels, NMDA/ AMPA/ KA channels,
stretch activated channels and other injury induced cation channels such as the TRPM-7 and acid sensing channels did not block this Ca\(^{2+}\) entry. Further, our results also demonstrate that there is a therapeutic window of opportunity of at least 1 h to block this current, decrease the elevated [Ca\(^{2+}\)], reverse END and prevent neuronal death. The development of a novel injury-induced Ca\(^{2+}\) permeable channel provides a molecular basis for the post-injury Ca\(^{2+}\) entry current responsible for producing END (Limbrick et al., 2003) and explains why many of the therapeutic trials employing conventional strategies to inhibit Ca\(^{2+}\) entry have not been effective in treating stroke. Activation of this injury induced Ca\(^{2+}\) permeable channel represents an early step in the cascade leading to excitotoxic neuronal death and offers a potential insight into developing novel therapeutic interventions to prevent brain injury from stroke.
Materials and Methods

Materials:

All the reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. mCPG (α-methyl-4-carboxyphenyl glycine), SKF-96365 (1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole), and TTX (tetrodotoxin) were obtained from Tocris (Ballwin, MO), Calbiochem (La Jolla, CA), and Alomone Labs (Jerusalem, Israel), respectively. DIDS (4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid), L-NAME (N (G)-nitro-L-arginine methyl ester) and AMILO (amiloride hydrochloride) were purchased from Sigma Chemical Co. MEM, L-glutamine, trypsin, penicillin-streptomycin, fetal bovine serum, and horse serum used in the tissue culture preparation were obtained from Gibco-BRL (Gaithersburg, MD).

Hippocampal neuronal cultures:

All animal use procedures were in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by Virginia Commonwealth University’s Institutional Animal Care and Use Committee. Cultured hippocampal neurons were prepared as described previously from 2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) with slight modifications (Sombati et al., 1991; Coulter et al., 1992; Limbrick et al., 2001). Cultures were fed thrice weekly with neuronal feed, maintained at 37°C in a 5% CO2/95% air atmosphere and utilized after 14 days in vitro.

Electrophysiology:

Whole cell patch clamp analyses were performed as described using an Axopatch 200A amplifier or an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA) in current clamp mode (Hamill et al., 1981; Coulter et al., 1992). Membrane potential was sequentially stepped
from -90 mV to +60 mV from a steady holding potential of -60 mV. Voltage steps were 50 ms in
duration and applied at a frequency of 0.2 Hz. Current responses were sampled at 20 kHz and
low-pass filtered at 1 kHz using a 4-pole Bessel filter (Frequency Devices, Haverhill, MA). The
recording solution contained (in mM) 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 CaCl₂ and 1
MgCl₂ (pH 7.3, 290 ± 10 mosM). The pipette solution contained (in mM) 140 K⁺ gluconate, 10
HEPES, 1.1 EGTA and 1 MgCl₂ (pH 7.2, 290 ± 10 mosM). Depending upon the experiments,
various inhibitors were included in the recording solution. In establishing the whole cell
configuration, gigaseal formation was verified, pipette capacitance canceled, and gentle suction
applied. Cells that required > 3 applications of suction for whole cell access were discarded.
Once whole cell access was established, whole cell capacitance was canceled. Series resistance
was generally 4-9 MΩ but was reduced by 75-80% using the compensation circuit of the
amplifier. Series resistance error was generally between 3-5 mV (but always < 9 mV).

Cell-attached single-channel recordings were performed as described (Hamill et al., 1981).
Fire polished, Slygard (Dow Corning Corp, MI) coated borosilicate glass pipettes had a
resistance of 7-10 MΩ when filled with recording solution containing (in mM): 145 NaCl, 2.5
KCl, 10 HEPES, 10 glucose, 2 CaCl₂ and 1 MgCl₂ (pH 7.3, 290 ± 10 mosM). Depending upon
the experiment various inhibitors were included in the pipette solution. Neurons were bath-
perfused with a “high-K⁺” solution in which the extracellular KCl concentration was raised from
2.5 to 40 mM to clamp the resting membrane potential near 0 mV. The patch was voltage
clamped at various voltages by applying a voltage of opposite sign to the patch pipette and the
recording was started. Current amplification was accomplished with an Axopatch 200A
amplifier and recorded on a VHS tape via a Neurocorder (Neurodata, NY) using Pclamp9 via
Digidata 1322A. Data were sampled at 10 kHz and filtered at 2 kHz.
Excitotoxic glutamate exposure:

Excitotoxic injury was induced as described previously (Choi et al., 1987; Michaels and Rothman, 1990; Sombati et al., 1991; Coulter et al., 1992) (Dubinsky, 1993; Limbrick et al., 1995). Bath application of glutamate was performed by gravity feed perfusion at a rate of 1 ml/min and solution changes were controlled through a six-valve perfusion system (Warner Instrument Corp., Hamden, CT). Glutamate (500 µM) was dissolved in recording solution and applied with 10µM glycine for 10 minutes. Glutamate washout was performed with control recording solution (2 mM CaCl₂), or Ca²⁺-free recording solution (0 mM CaCl₂; omitting CaCl₂ but contained no Ca²⁺ chelator), or high-Ca²⁺ recording solution (10 mM CaCl₂; equimolar replacement of NaCl), or Na⁺-free recording solution (equimolar substitution of N-methyl-D-glucamine (NMDG) chloride for NaCl).

Calcium Microfluorometry:

Fura-2 acetoxyethyl ester was loaded in the neurons and then transferred to a heated stage (37°C) of an Olympus IX-70 inverted microscope coupled to an ultra-high-speed fluorescence imaging system (Olympus/ Perkin-Elmer) (Dubinsky, 1993; Limbrick et al., 1995). Ratio images were acquired by using alternating excitation wavelengths (340/380 nm) with a filter wheel (Sutter Instruments, Novato, CA) and fura filter cube at 510/540 nm emissions with a dichroic mirror at 400 nm. Image pairs were captured and digitized every 15s, and the images at each wavelength were averaged over four frames and corrected for background fluorescence by imaging a non-indicator loaded field.
Cell death assay:

Neuronal death was characterized using the Vybrant Apoptosis Assay Kit #3 (Molecular Probes, Eugene, OR) (Raza et al., 2001). Cells were treated with FITC annexin-V 5 µl /100 µl of total volume and 1 µl of 100 µg/ml of PI solution. After 15 min, 400 µl of 1X annexin-binding buffer was added and cells were visualized. Fraction of neuronal death was calculated as:

\[ \text{Fraction Dead} = \frac{(\text{Dead}_{\text{treat}} - \text{Dead}_{\text{control}})}{\text{Dead}_{\text{glutamate}}} \]

Data Analysis:

Averaging the final 17 ms of each step to minimize any effect of 60 Hz noise generated steady-state current-voltage (I-V) relationships. Permeability ratios were calculated using the Goldman-Hodgkin-Katz (GHK) voltage equation. Statistical differences in the magnitude of current responses or END potentials or cell-death were tested using a One-Way Analysis of Variance followed by post-hoc Tukey test. Single channel analyses were accomplished using ClampFit 9. Statistical tests were run using SigmaStat 2.0 and graphs were generated with SigmaPlot 8.0 (SPSS Inc, Chicago).
Results

Excitotoxic glutamate injury produces a persistent inward excitotoxic injury current (I_{EIC})

To investigate the molecular basis of END, whole cell recordings were conducted on control and glutamate injured neurons in the presence of a mixture of Ca^{2+} entry inhibitors that were demonstrated to inhibit voltage gated Ca^{2+} channels (nifedipine, 5 µM), NMDA channel (MK-801, 10 µM), AMPA/ KA channels (CNQX, 10 µM), the TRP channel family and stretch activated ion channels (GdCl3, 10 µM) (Sattler et al., 1998; Aarts et al., 2003). Recordings in the presence this Ca^{2+} entry inhibitor cocktail has been shown by our laboratory and others (Sattler et al., 1998; Aarts et al., 2003) to inhibit the standard mechanisms of Ca^{2+} entry and provides the ability to investigate the presence or absence of Ca^{2+} entry following excitotoxic injury.

Control neurons exhibited a membrane potential of -56.7 ± 1.3 mV and mean input resistance of 240.7 ± 32.5 MΩ (n=10, Table 1). Whole-cell voltage-clamp recordings from control neurons in the presence of cocktail of Ca^{2+} entry inhibitors showed no steady state inward currents (Fig.1A). Outward currents consistent with activation of voltage dependent K^+ channels were observed in control neurons at voltages positive to -40 mV and were sensitive to K^+ channel blockers (4-AP, charybdotoxin). In contrast, excitotoxic glutamate injured neurons in the presence of Ca^{2+} entry inhibitors mixture manifested END (Sombati et al., 1991; Coulter et al., 1992) and exhibited a membrane potential of -14.0 ± 1.6 mV and an input resistance of 26.6 ± 3.4 MΩ, that were statistically different from control neurons (p < 0.001 for both, Table 1). Neurons in END revealed a persistent inward excitotoxic injury current (I_{EIC}) (Fig. 1A). We contend that this persistent inward current represents the basis of the Ca^{2+} paradox. The peak inward current for the END conditions in the presence of Ca^{2+} entry inhibitors was -3934.6 ± 635.5 pA (n=12). Steady state outward currents in excitotoxic glutamate injured neurons were
identical in magnitude to those observed in control neurons. The net I-V relationships (Aarts et al., 2003) for glutamate-injured neurons (total EIC minus control; Fig. 1B) were studied in the remaining experiments. This net inward current had a reversal potential (E_{rev}) of ~ + 40 mV.

Cell-attached single-channel recordings from glutamate-injured neurons demonstrated distinct openings and closings of an ion channel that existed in the presence of Ca^{2+} entry inhibitors and corresponded to the appearance of I_{EIC} during END (Fig. 1C). This channel exhibited an essentially linear I-V relationship with a unitary conductance of 49.5 ± 5.2 pS (Fig. 1D) (n=7). Conversely, control neurons demonstrated no channel activity in the presence of Ca^{2+} entry inhibitors.

We recently showed that END could be reversed by application of 100 µM GdCl₃ or removal of [Ca^{2+}]_e after glutamate excitotoxicity (Limbrick et al., 2003). To investigate if activation of I_{EIC} and the observed channel were responsible for mediating END, we recorded whole-cell and single-channel currents under these conditions (Limbrick et al., 2003). Application of Gd^{3+} (100 µM) or removal of [Ca^{2+}]_e in the post-glutamate END period abolished the inward current and restored the I-V profile identical to that of a control neuron (Fig. 1E, F). In addition, Gd^{3+} treated neurons (n=14) and neurons recorded in absence of [Ca^{2+}]_e (n=11) had a mean membrane potential of -52.4 ± 3.9 mV and -49.6 ± 3.4 mV respectively. The mean input resistance for these two conditions were 246.3 ± 41.3 MΩ and 257.4 ± 39.7 MΩ. These parameters were not significantly different from control neurons (p = 0.075 and 0.745 respectively, Table 1). Both the inward current and channel activity were not voltage dependent over the range of voltages studied. Furthermore, the current was not inhibited by cocktail of Ca^{2+} entry inhibitors, ruling out the involvement of voltage dependent Ca^{2+} channels. Taken together,
these results indicate that excitotoxic glutamate exposure produces END and activates a novel channel activity that carries $I_{\text{EIC}}$ in injured neurons.

**Calcium ions are the primary permeant ions for $I_{\text{EIC}}$ and the $I_{\text{EIC}}$ channel**

To confirm the ionic basis of END, $I_{\text{EIC}}$ and the channel mediating $I_{\text{EIC}}$, major cations were sequentially replaced in the recording solution. Equimolar substitution of NaCl with NMDG failed to reverse END (Fig. 2A), did not affect the magnitude of $I_{\text{EIC}}$ (Fig. 2B) and had no effect on channel activity (Fig. 2C). In addition removal of Na$^+$ did not affect the membrane potential (-9.4 ± 2.0 mV) or input resistance (38.3 ± 5.2 MΩ), when compared to END neurons ($p = 0.101$ and $0.093$, respectively, $n = 16$, Table 1). These results indicate that Na$^+$ influx did not significantly contribute to $I_{\text{EIC}}$.

Removing [Ca$^{2+}$]$e$ following glutamate excitotoxicity completely reversed END (Fig. 2A), abolished $I_{\text{EIC}}$ (Fig. 2B) and also abolished single channel events (Fig. 2C). Neurons subjected to Ca$^{2+}$ free conditions after excitotoxic injury exhibited a mean membrane potential of -49.6 ± 3.4 mV and an input resistance of 257.4 ± 39.7 MΩ, that were not significantly different from control neurons ($p = 0.075$ and $0.745$, respectively, $n = 11$, Table 1). Permeability ratios calculated from the GHK equation (Hille, 2001), using the steady state $E_{\text{rev}}$, indicated that $I_{\text{EIC}}$ is mediated by a highly selective Ca$^{2+}$ conductance that is 50 times more permeable to Ca$^{2+}$ than Na$^+$ ($P_{\text{Ca}}:P_{\text{Na}}= 12.5:0.25$).

To further establish that Ca$^{2+}$ influx was responsible for END and was the major ion permeating the $I_{\text{EIC}}$ channel, we measured [Ca$^{2+}$]$i$ using Fura-2 imaging. Substituting NaCl with NMDG had no effect on [Ca$^{2+}$]$i$. Conversely, removing [Ca$^{2+}$]$e$ reversed the elevated [Ca$^{2+}$]$i$, observed after glutamate excitotoxicity (Fig. 2D).
The Ca\textsuperscript{2+} dependency of I\textsubscript{EIC} was then evaluated by investigating the effects of variable [Ca\textsuperscript{2+}]\textsubscript{e} on magnitude of I\textsubscript{EIC}. The net peak inward currents reduced from -3932.3 ± 635.5 pA to -1119.7 ± 327.4 pA at -90 mV during 2 and 0.5 mM [Ca\textsuperscript{2+}]\textsubscript{e} (Fig. 2E) and also caused a significant shift in the net E\textsubscript{rev} from +40.5 ± 2.2 to -20.6 ± 2.0 mV, respectively. This value agreed with the theoretical E\textsubscript{rev} value of ~ -22 mV determined by the Nernst equation for 0.5 mM [Ca\textsuperscript{2+}]\textsubscript{e} and resulted in the predicted linear relationship of E\textsubscript{rev} versus log\textsubscript{10} [Ca\textsuperscript{2+}]\textsubscript{e} (Fig. 2F). The membrane potential for neurons in 0.5 mM CaCl\textsubscript{2} was -25.9 ± 4.6 mV, and the input resistance was 59.1 ± 6.9 M\textOmega. These values represent significant changes from those measured in 2 mM CaCl\textsubscript{2} for END (p = 0.013, p<0.001 respectively, n = 9, Table 1).

Conversely, increasing [Ca\textsuperscript{2+}]\textsubscript{e} from 2 mM to 10 mM resulted in an increased net peak inward current of -5590.8 ± 457.8 pA (Fig. 2E) and resulted in a membrane potential of -1.5 ± 2.4 mV and an input resistance of 22.9 ± 1.7 M\textOmega. Increasing [Ca\textsuperscript{2+}]\textsubscript{e} to 10 mM caused a shift in the net E\textsubscript{rev} from +40.5 ± 2.2 to +50.1 ± 4.2 mV. The observed shift of E\textsubscript{rev} with 10 mM CaCl\textsubscript{2} was slightly less than the predicted E\textsubscript{rev} (~ +62 mV) and may be due to saturation of E\textsubscript{rev} when plotted against log\textsubscript{10} [Ca\textsuperscript{2+}]\textsubscript{e} (Fig. 2F). This observation is consistent with the concept of permeant ion rectification, where saturation of I\textsubscript{EIC} occurs with respect to [Ca\textsuperscript{2+}] (Hille, 2001). Thus, I\textsubscript{EIC} followed Nernstian predictions in response to changes in [Ca\textsuperscript{2+}]\textsubscript{e}.

Taken together, the following evidence supports the conclusion that the injury induced Ca\textsuperscript{2+} permeable channel is responsible for carrying the I\textsubscript{EIC} that mediates END: 1) activation of the ion channel in the post-glutamate END period coincides with the appearance of I\textsubscript{EIC}; 2) END, I\textsubscript{EIC} and the Ca\textsuperscript{2+} permeable channel demonstrate identical ionic selectivity and are abolished in the absence of [Ca\textsuperscript{2+}]\textsubscript{e}; 3) END, I\textsubscript{EIC} and the Ca\textsuperscript{2+} permeable channel all manifest identical insensitivity to blockade by Ca\textsuperscript{2+} entry inhibitors mixture; 4) They all are inhibited by higher
concentrations of Gd$^{3+}$. This data provides the first direct evidence of a novel Ca$^{2+}$ permeable channel that can clarify the Ca$^{2+}$ paradox and explain the persistent entry of Ca$^{2+}$ despite the use of Ca$^{2+}$ entry inhibitors.

**Traditional routes of Ca$^{2+}$ entry or internal stores do not mediate $I_{EIC}$**

We employed extensive pharmacological studies to establish that the $I_{EIC}$-Ca$^{2+}$ permeable channel represented a new route of Ca$^{2+}$ entry. END neurons are characterized by membrane potentials of approximately -15 to -20 mV. Activation of voltage-gated Ca$^{2+}$ channels (VGCC) is expected to occur at these potentials. Thus, the observed injury mediated channel activity could be due to the activation of the voltage-gated Ca$^{2+}$ channels. In order to investigate contribution of VGCCs to END, a combination of effective concentrations of L-type VGCC antagonist (nifedipine, 5 µM), N-type VGCC antagonist (Ω-conotoxin GVIA, 1 µM), P/Q-type VGCC antagonist (Ω-conotoxin MVIIC, 100 nM) or T-type VGCC antagonist (ethosuximide, 1 mM) along with other components of Ca$^{2+}$ entry inhibitor mixture (such as MK-801 and CNQX) was used to wash out glutamate. Since alterations in VGCC gating could also occur during or after excitotoxic glutamate exposure, we included 200 µM CoCl$_2$ in the inhibitor combination. This combination had no effect on END membrane potentials suggesting that the VGCCs are not mediating the injury induced ion channel activity (Fig. 3A). Most of the cation channels including the stretch activated channels and other Ca$^{2+}$ channels are completely or maximally blocked at GdCl$_3$ concentrations of 10 µM (Caldwell et al., 1998). However, this concentration did not inhibit END nor decrease $I_{EIC}$ or single channel activity after glutamate excitotoxicity. Thus, while use of 10 µM GdCl$_3$ inhibited these other channels under our conditions, but it had no effect on END. In fact, END and related physiological processes were abolished only when the Gd$^{3+}$ concentration was raised to 100 µM. This is a very large difference in concentration and
provides a major distinction. This dose dependent effect of Gd$^{3+}$ clearly differentiates the Ca$^{2+}$ channel observed in our study from the traditional GdCl$_3$ sensitive Ca$^{2+}$ channels previously identified. In addition, voltage-gated Ca$^{2+}$ channels undergo inactivation rapidly after the onset of depolarization. But we observed a persistent channel activity for prolonged period in the depolarized END phase. Moreover, VGCC’s are characterized by low single channel conductance. Taken together, we can conclude that VGCCs are not the mediators of the glutamate injury induced Ca$^{2+}$ permeable channel.

Given the high Ca$^{2+}$ permeability of this channel and a requirement of NMDA for channel activation one probable candidate is the NMDA receptor gated channel. We used NMDA-receptor antagonists MK-801 (10 µM) and APV (50 µM) either alone or in combination. MK-801 is a voltage-dependent blocker of NMDA-receptor ion channel. Thus its ability to block is limited under depolarized (END) condition. APV is a competitive antagonist and its ability to block NMDA-receptor depends upon the period of glutamate exposure. Our observations that both the competitive (APV) and non-competitive (MK-801) pharmacological inhibitors to NMDA channel do not block the post-glutamate injury induced channel activity (Fig. 1; experiments were performed in the presence of Ca$^{2+}$ entry inhibitor mixture that contained MK 801) or END potentials (Fig. 3A) provide strong evidence that NMDA receptor does not mediate the injury-induced current. Similarly the inability of AMPA/KA receptor antagonists (CNQX, 10 µM; NBQX, 10 µM) or the metabotropic glutamate receptor antagonist (mCPG, 250 µM), to block the injury induced channel activity suggests that other glutamate receptor subtypes do not represent the molecular basis for excitotoxic glutamate activated channel (Fig. 3A).

The bivalent metal cation zinc (Zn$^{2+}$) is known to regulate a number of ligand-gated, voltage-gated, and nonselective cation channels, several of which gate Ca$^{2+}$ entry into the
neurons (Christine and Choi, 1990; Chen et al., 1997). In particular Zn\(^{2+}\) blocks NMDA and AMPA receptors and most types of VGCC’s. However Zn\(^{2+}\) at a lower 5-µM concentration, a concentration that was also greater than the dissociation constant of Zn\(^{2+}\) at the NMDA receptor Zn\(^{2+}\) site (Christine and Choi, 1990), had no effect on the post-glutamate membrane potential (Fig. 3A). Conversely, inclusion of ZnCl\(_2\) (500 µM) following removal of glutamate allowed a rapid and complete repolarization to resting membrane potential (Fig. 3B). Thus the ability of ZnCl\(_2\) to reverse END was independent of its effects on NMDA receptors.

Other mechanisms of ionic entry were also tested. Inhibiting the forward and reverse mode of Na\(^+\)/ Ca\(^{2+}\) exchanger (bepridil, 50 µM or [Na\(^+\)]\(_o\) removal), chloride channels (DIDS, 100 µM), and stretch receptors channels (GdCl\(_3\), 10 µM) also had no effect on diminishing the END potentials after glutamate excitotoxicity (Fig. 3A). In addition, the strict ionic selectivity, the differential permeability of I\(_{EIC}\) and the fact that changes in [Ca\(^{2+}\)]\(_i\) could be measured throughout our experiments demonstrated that I\(_{EIC}\) was not the result of a non-specific ion leak or membrane disruption following glutamate excitotoxicity.

Studies were also done to evaluate the contribution of internal Ca\(^{2+}\) stores that are known to play a major role in [Ca\(^{2+}\)] homeostasis (Duchen, 2000). Blocking store operated Ca\(^{2+}\) channels (SKF-96365, 10 µM), ryanodine receptors (dantrolene, 20 µM) or IP\(_3\) receptors (heparin, 2mg/ml) or intracellular Ca\(^{2+}\) release inhibitor (thapsigargin, 1 µM) (data not shown) had no effect on reducing END. Compensating for mitochondrial injury by addition of an ATP regenerating system (4 mM ATP and 22 mM phosphocreatinine) or by inhibiting mitochondrial Ca\(^{2+}\) release (rhodamine, 10 µM, data not shown) also had no effect on reducing END (Fig. 3A). Indeed mitochondria maintain their resting level for Ca\(^{2+}\) for about 45-mins after glutamate excitotoxicity, even in the face of rising cytosolic Ca\(^{2+}\) levels (Bano et al., 2005). Moreover,
Mitochondrial respiration is retained for a relatively long time in cerebellar neurons undergoing excitotoxicity (Jekabsons and Nicholls, 2004) and the final mitochondrial Ca\(^{2+}\) deregulation and the permeability transition were downstream rather than upstream of the secondary Ca\(^{2+}\) overload following glutamate excitotoxicity (Bano et al., 2005). Although intracellular Ca\(^{2+}\) stores and other cation conductances may play role in ischemia induced [Ca\(^{2+}\)]\(_i\) accumulation, our results demonstrate that I\(_{EIC}\) is responsible for majority of the early Ca\(^{2+}\) influx after glutamate excitotoxicity.

**Neuroprotection with Gadolinium: Evidence that I\(_{EIC}\) accounts for the Ca\(^{2+}\) paradox**

To test whether activation of I\(_{EIC}\) could explain the Ca\(^{2+}\) paradox, we investigated if blocking this channel could prevent neuronal death after glutamate excitotoxicity. Treatment with 100\(\mu\)M Gd\(^{3+}\) produced a significant reduction in the number of annexin positive cells (Fig. 3C and 4) and also conferred neuroprotection when administered 1-h after excitotoxic injury (Fig. 3D). Further, with Gd\(^{3+}\) intervention, up to 50% neuroprotection was observed even out to 2-h after glutamate excitotoxicity (Fig. 3D).

It has been suggested that activation of nNOS via Ca\(^{2+}\) coming specifically from NMDA receptors plays a role in neuronal cell death. This observation is based on the findings that blocking formation of NO using NOS inhibitors attenuates glutamate-induced neuronal death (Yamauchi et al., 1998). However, it has been shown that this effect is concentration and time dependent (Vige et al., 1993). Thus, addition of NG-nitro-L-arginine (100 \(\mu\)M) to the medium either 5 min prior to and during L-glutamate exposure (500 \(\mu\)M, 5 min) decreased the amino acid-induced neurotoxicity only by 20%. But, when added 5 min before L-glutamate and kept in contact with neurons for the following 24 h after glutamate removal, NG-nitro-L-arginine (100 \(\mu\)M) antagonized the glutamate-induced neurotoxicity by more than 70% (Vige et al., 1993).
Several research groups have reported that inhibition of NOS following a 5-10 min exposure to 300-500 µM NMDA did not attenuate neuronal death in primary cortical cultures from rat and mouse (for example: (Hewett et al., 1993)). These results from several prominent groups in the neuronal death field clearly demonstrate that NOS inhibition does not attenuate neuronal cell death when the inhibition is administered immediately after a neurotoxic insult. However, pretreatment with various NOS inhibitors can be successful in reducing excitatory amino acid neurotoxicity (for example: (Yamauchi et al., 1998)). In our hands post treatment with L-NAME was not effective in blocking neuronal cell death. In addition, we found that when L-NAME was given during and after glutamate insult, only a small but statistically insignificant decrease in neuronal cell death was observed (Fig. 3C). This is consistent with what is reported in the literature. NOS inhibition only significantly protects neurons when it is inhibited before the injury with glutamate injury.

Fura-2 imaging experiments demonstrated that injured neurons restored excitotoxic glutamate induced elevated [Ca\(^{2+}\)]\(_i\) to basal levels for up to 1-h after removal of [Ca\(^{2+}\)]\(_c\) (Fig. 3E and 4). However beyond this time point the percentage of neurons that could restore basal [Ca\(^{2+}\)]\(_i\) levels started to decrease despite absence of [Ca\(^{2+}\)]\(_c\). These results demonstrate that there is a window of opportunity for neuroprotection of up to 1-h after glutamate excitotoxicity during which it is possible to reverse the increased [Ca\(^{2+}\)]\(_i\) while the I\(_{EIC}\) channel is the major source of elevated [Ca\(^{2+}\)]\(_i\). In addition, as shown in Fig. 1A, injured neurons revealed I\(_{EIC}\) despite the presence of Ca\(^{2+}\) entry inhibitors. Thus this data provide an explanation for the Ca\(^{2+}\) paradox by demonstrating that blocking I\(_{EIC}\) Ca\(^{2+}\)-permeable channel was able to prevent Ca\(^{2+}\) entry inhibitor resistant Ca\(^{2+}\) entry, reverse END and inhibit cell death.
Comparison of $I_{\text{EIC}}$ to other injury-induced cation currents

It is important to compare $I_{\text{EIC}}$ to other nonselective cation currents that have been observed in association with different types of neuronal injury. Recently, TRPM-7 channels were shown to carry an anoxia-induced cation current ($I_{\text{OGD}}$) in cortical neurons (Aarts et al., 2003). Two hours of anoxia induced $I_{\text{OGD}}$ which was inhibited by 300 µM L-NAME or 10 µM GdCl$_3$ and increased in magnitude upon $[\text{Ca}^{2+}]_e$ removal (Aarts et al., 2003). In contrast, induction of $I_{\text{EIC}}$ was rapid and treatments with L-NAME or 10 µM GdCl$_3$ during and after glutamate excitotoxicity failed to reverse END (Fig. 3A), inhibit $\text{Ca}^{2+}$ influx (data not shown) or prevent neuronal death (Fig. 3C). Moreover, $I_{\text{EIC}}$ decreased in magnitude upon $[\text{Ca}^{2+}]_e$ removal. Thus, based on the kinetic characteristics and pharmacological comparisons it appears that TRPM-7 channels are not responsible for mediating $I_{\text{EIC}}$.

Acidosis from ischemia induces the activation of amiloride sensitive high Na$^+$-low Ca$^{2+}$ permeable cation channels (ASIC’s) (Xiong et al., 2004). Amiloride (100 µM) or its derivative, bepridil (50 µM) had no significant effect on END (Fig. 3A), failed to block $\text{Ca}^{2+}$ influx (data not shown) and did not prevent cell death after glutamate excitotoxicity (Fig. 3C). Furthermore, the activation of ASIC’s was unlikely because our perfusion conditions prevented the development of acidic conditions, demonstrating that ASIC’s are not responsible for mediating $I_{\text{EIC}}$.

Excitotoxic injury with NMDA can also induce a post exposure current ($I_{\text{pe}}$) that was shown not selective for $\text{Ca}^{2+}$ ($P_{\text{Ca}}:P_{\text{Na}}=7:1$) and not altered by removal of $[\text{Ca}^{2+}]_e$ in acutely isolated hippocampal neurons (Chen et al., 1997). In contrast, steady state $I_{\text{EIC}}$ has a high Ca$^{2+}$ selectivity ($P_{\text{Ca}}:P_{\text{Na}}=50:1$) and is abolished upon omission of $[\text{Ca}^{2+}]_e$, demonstrating that $I_{\text{pe}}$ is not responsible for mediating $I_{\text{EIC}}$. 


Taken together, these results demonstrate that TRPM-7, ASIC or I_pe are not significantly contributing to the initial Ca^{2+} entry or END during the first hour of the post-glutamate treatment paradigm and that I_{EIC} is a unique Ca^{2+} current which is mediated by a novel Ca^{2+} permeable channel.
Discussion

This study demonstrates that glutamate excitotoxicity activates a previously undetected Ca\(^{2+}\) permeable channel in cultured hippocampal neurons that carries the injury-induced current (I\(_{\text{EIC}}\)), primarily responsible for the initial sustained increases in [Ca\(^{2+}\)], following neuronal injury that maintains END and ultimately leads to neuronal death. Inhibiting this I\(_{\text{EIC}}\)-Ca\(^{2+}\) permeable channel within the window of opportunity after glutamate excitotoxicity reversed END, blocked Ca\(^{2+}\) entry and prevented delayed neuronal death. To our knowledge the development of the Ca\(^{2+}\) entry inhibitor resistant I\(_{\text{EIC}}\)-Ca\(^{2+}\) permeable channel following glutamate excitotoxicity provides the first molecular insight into the Ca\(^{2+}\) paradox and explains why many of the clinical trials employing conventional strategies to inhibit Ca\(^{2+}\) entry have not been effective in treating excitotoxic neuronal injury and stroke (Ikonomidou and Turski, 2002; Wahlgren and Ahmed, 2004).

Numerous studies have demonstrated a causal relationship between ischemia induced neuronal death and [Ca\(^{2+}\)]\(_i\) accumulation (Kristian and Siesjo, 1998). Thus, a critical question relates to the source of this Ca\(^{2+}\). While intracellular Ca\(^{2+}\) stores and other cation conductances may play some role in ischemia induced Ca\(^{2+}\) elevations, our data demonstrate that the activation of the I\(_{\text{EIC}}\)-Ca\(^{2+}\) permeable channel is responsible for the majority of the Ca\(^{2+}\) influx during the first hour after glutamate excitotoxicity. During this 1-h time window, intervention with Gd\(^{3+}\) or removal of [Ca\(^{2+}\)]\(_e\) can restore elevated [Ca\(^{2+}\)]\(_i\). However, beyond this time point, in addition to I\(_{\text{EIC}}\) other irreversible ionic mechanisms are activated, including Ca\(^{2+}\) release from mitochondria, from intracellular stores and from alterations in Ca\(^{2+}\) homeostatic mechanisms such that it is no longer possible to lower the elevated [Ca\(^{2+}\)]\(_i\) despite removal of [Ca\(^{2+}\)]\(_e\).
The activation, but not the maintenance of END and the $I_{EIC}$-$Ca^{2+}$ permeable channel is dependent upon NMDA receptor activation during excitotoxicity (Limbrick et al., 2003) since the presence of the NMDA channel inhibitor, MK-801, during glutamate injury prevents cell death and END. In contrast, MK-801 administered after excitotoxic injury did not prevent cell death, inhibit $Ca^{2+}$ entry during END or inhibit the $I_{EIC}$-$Ca^{2+}$ permeable channel. In addition, depolarization induced $Ca^{2+}$ entry alone did not produce END (Coulter et al., 1992). Treatments with high concentrations of potassium chloride or substitution of $[Ca^{2+}]_e$ with $[Ba^{2+}]_e$ during glutamate excitotoxicity both caused neuronal depolarization but did not cause the induction of END (Coulter et al., 1992). These findings indicate that the $I_{EIC}$-$Ca^{2+}$ permeable channel is activated by an NMDA/$Ca^{2+}$ mechanism. Since $I_{EIC}$ could be observed immediately following 10-min glutamate stimulation, it is unlikely that $I_{EIC}$ represents a newly synthesized channel, since this may be too rapid a time frame for de-novo protein synthesis and insertion into the membrane. It is more likely that excitotoxic stimulation would trigger second messenger cascades leading to post-translational modifications of existing membrane proteins or activation of a dormant channel to activate $I_{EIC}$. Indeed NMDA dependent changes in protein phosphorylation (Churn et al., 1995; Durkin et al., 1997), protease activity such as the calpains (Minger et al., 1998; Simpkins et al., 2003) and numerous other second messenger effects have been reported during glutamate excitotoxicity. Such alterations in key proteins or enzyme activities could result in the activation of the $I_{EIC}$-$Ca^{2+}$ permeable channel. Future studies are planned to investigate these possibilities and elucidate the molecular basis of the activation of the $I_{EIC}$-$Ca^{2+}$ permeable channel using both in vitro and in vivo models of stroke and brain injury.

Protracted $Ca^{2+}$ increases upon excitotoxicity persist well beyond the period of glutamatergic injury (Dubinsky, 1993; Limbrick et al., 1995) and cause diverse
pathophysiological changes including generation of free radicals, neuronal acidity, activation of proteases all of which can trigger neurodegenerative processes (Lipton, 1999). These sustained elevations in [Ca$^{2+}$], represent a prolonged imbalance in Ca$^{2+}$ homeostasis and correlate with subsequent excitotoxic neuronal death (Dubinsky, 1993; Limbrick et al., 1995). This [Ca$^{2+}$], deregulation could result either from a persistent influx of [Ca$^{2+}$], and/or from sustained impairment of neuronal Ca$^{2+}$ sequestration/extrusion mechanisms. Indeed recent discoveries that TRPM-7 (Aarts et al., 2003) and ASIC’s (Xiong et al., 2004) allow for Ca$^{2+}$ entry upon hypoxia-ischemia and that the plasma membrane Na$^{+}$/Ca$^{2+}$ exchanger undergoes cleavage upon excitotoxicity (Bano et al., 2005) suggests that both these possibilities exist. These observations demonstrate the importance of elucidating the mechanisms underlying the sustained [Ca$^{2+}$], elevations following glutamate excitotoxicity.

Earlier attempts to elucidate the basis of the post excitotoxic injury-induced Ca$^{2+}$ paradox indicated that an influx of extracellular Ca$^{2+}$ was responsible for END (Limbrick et al., 2003). However that study didn’t identify the source or nature of this post-injury Ca$^{2+}$ entry. The data presented here is a major advance over this earlier work and demonstrates that a unique Ca$^{2+}$ current is activated upon excitotoxic injury that is the molecular basis for the influx of extracellular Ca$^{2+}$ responsible for END. This paper not only documents the existence of this novel Ca$^{2+}$ permeable channel, but also provides a careful pharmacological characterization of the channel and differentiates it from other types of calcium channels reported in the literature. Our findings demonstrate that the I$_{EIC}$-Ca$^{2+}$ permeable channel is activated by glutamate excitotoxicity and blocking its activity after the excitotoxic insult prevents [Ca$^{2+}$], accumulation and neuronal cell death. These findings suggest that activation of I$_{EIC}$-Ca$^{2+}$ permeable channel could represent an early step in the genesis of the injury induced [Ca$^{2+}$], plateau. The possible
identification of a novel molecular target compliant to pharmacological manipulations opens exciting avenues for the treatment of acute and chronic neurological disorders associated with glutamate excitotoxicity. Elucidation of the $I_{EIC}$ may provide a new target for a significant extension of the therapeutic window to prevent neuronal death in stroke and offers new hope in the search for novel agents to treat stroke and excitotoxic brain injury.
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Evidence that the early loss of membrane protein kinase C is a necessary step in the


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Footnotes:

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**Fig. 1** Induction of an inward current, $I_{EIC}$, following glutamate excitotoxicity. (A) Current traces and (B) I-V relationship from a representative control (n=10) or END (n=12) neuron. Note the large inward current, $I_{EIC}$, observed in END neuron that is not blocked by standard Ca$^{2+}$ entry inhibitors. (C) Representative trace showing induction of single-channel activity in cell-attached patches and I-V relationship from excitotoxic glutamate injured neurons. Glutamate-injured neurons demonstrated robust single channel activity in the presence of cocktail of Ca$^{2+}$ entry inhibitors. No single-channel events were observed in control neurons (n=6). (D) The excitotoxic glutamate-induced channel had a slope conductance of 48.5 ± 4.2 pS. (E) I-V curves and (F) single-channel activity for omission of [Ca$^{2+}$]$_e$ or treatment with Gd$^{3+}$ (100 µM) (n=7). These treatments abolished $I_{EIC}$-channel activity in the excitotoxic glutamate-injured neurons.

**Fig. 2** $I_{EIC}$ is highly selective for Ca$^{2+}$. (A) Representative current clamp traces show that [Ca$^{2+}$]$_e$ but not [Na$^+$]$_e$ was required for the maintenance of END. (B) I-V curves for omission of [Ca$^{2+}$]$_e$ or [Na$^+$]$_e$. Omission of Ca$^{2+}$ (n=11), but not Na$^+$ (n=12) after glutamate excitotoxicity abolished $I_{EIC}$ (n=10). (C) The excitotoxic glutamate injury-induced ion channel is Ca$^{2+}$ selective. Representative single-channel traces demonstrate no channel events in the absence of Ca$^{2+}$ in the pipette (n=6) while omission of Na$^+$ maintained channel activity (n=6). (D) Direct measurements of [Ca$^{2+}$]$_i$, expressed as fura-2 ratios, demonstrate that removal of [Ca$^{2+}$]$_e$ (n=8), but not [Na$^+$]$_e$ (n=7) reversed elevated [Ca$^{2+}$]$_i$ (n=9) following glutamate excitotoxicity. (E) I-V curves for changes in [Ca$^{2+}$]$_e$. Lowering [Ca$^{2+}$]$_e$ decreased the magnitude of $I_{EIC}$ (n=9). Conversely, raising [Ca$^{2+}$]$_e$ increased the magnitude of $I_{EIC}$ (n=12). (F) Varying [Ca$^{2+}$]$_e$ caused shifts in the net $E_{rev}$ according to Nernstian predictions.
**Fig. 3** Evidence that $I_{IEIC}$ accounts for the Ca$^{2+}$ paradox of excitotoxic neuronal death. (A) Mean membrane potentials for control (CTRL), END and END + test agents conditions: CEI (Ca$^{2+}$ entry inhibitors: MK-801, CNQX, nifedipine and 10 µM Gd$^{3+}$ along with Ω-conotoxin and ethosuximide), mCPG (α-methyl-4-carboxyphenyl glycine), TTX (tetrodotoxin), BEP (bepridil), DIDS (4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid), SKF (SKF-96365: 1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole), ATP, L-NAME (N(G)-nitro-L-arginine methyl ester), AMILO (amiloride), 10 Gd$^{3+}$, and 5 Zn$^{2+}$ (n=6). Treatment with these agents after glutamate excitotoxicity didn’t abolish END. (B) Treatment after glutamate injury with 0 Ca$^{2+}$, 100 Gd$^{3+}$ and 500 Zn$^{2+}$, but not 0 Na$^{+}$ (n=5) inhibited END. (C) Treatment with Gd$^{3+}$ (100 µM) was neuroprotective and prevented neuronal cell death. Conversely, inhibition of TRPM-7 (L-NAME, 10 µM Gd$^{3+}$) or ASIC’s (amiloride- AMILO) had no effect on fraction of dead cell count. (D) And (E) Blockade of the $I_{IEIC}$-Ca$^{2+}$ permeable channel at different times after glutamate excitotoxicity demonstrates an the extension of the therapeutic window for preventing cell death and the post-injury [Ca$^{2+}$], plateau. Glutamate-injured neurons were treated with (D) 100 µM Gd$^{3+}$ or (E) 0-Ca$^{2+}$ solutions starting at 0-h and up to 4-h after glutamate excitotoxicity. The percentage of neurons undergoing cell death or returning to basal [Ca$^{2+}$], ratios were measured (n=6). Data expressed as mean ± SEM. For A and B asterisks denote differences from control and from GLU for C, D and E (*p< 0.05). [Color scheme: black = control; white = END/glutamate; and gray = inhibitors].

**Fig. 4** Effect of Ca$^{2+}$ removal and 100 µM GdCl$_3$ treatment on [Ca$^{2+}$], dynamics and neuronal death after excitotoxic glutamate injury. The right side panels show fluorescent Fura-2 Ca$^{2+}$ images from the same field of neurons before glutamate exposure (top panel), during END (middle panel) and upon removal of [Ca$^{2+}$]$_e$ (bottom panel). As depicted in the fluorescent scale
bar, blue/green color indicates a low 340/380 ratio that corresponds to low \([\text{Ca}^{2+}]_i\). Conversely, red indicates a high 340/380 ratio that corresponds to elevated \([\text{Ca}^{2+}]_i\). As shown in the fluorescent images, control panel (top) is characterized by predominantly bluish-green neurons. Upon glutamate exposure and during END majority of the neurons show bright red color in the cytoplasm, which corresponds to elevated \([\text{Ca}^{2+}]_i\). When \([\text{Ca}^{2+}]_e\) is removed after excitotoxic injury, \(I_{\text{EIC}}\) is blocked, which results in restoration of elevated \([\text{Ca}^{2+}]_i\) now characterized by greenish color in the majority of neurons (bottom panel). The right panels show fluorescent images from a cell death assay using the Annexin and propidium iodide (PI) stains. Annexin-FITC identifies apoptotic cells by binding to exposed phosphatidylserine and emitting a green fluorescence. PI is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. Viable neurons are the phase-bright neurons. The top panel on right-side shows a fluorescent image from a control plate stained with Annexin-PI. There are only a few neurons emitting green or red fluorescence, indicating low cell death. END (middle panel) is characterized by a preponderance of neurons emitting green/ red fluorescence, showing significant cell death. However, treatment with 100 mM GdCl\(_3\) (bottom panel) after glutamate injury blocks END and attenuates neuronal death as characterized by only a few neurons emitting green fluorescence, demonstrating significant neuroprotection.
Table 1. Effects of post-glutamate extracellular ionic replacement or drug addition on post-glutamate membrane potential and input resistance

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>Number Of Cells</th>
<th>Membrane Potential (mV)</th>
<th>Input Resistance (MΩ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>-56.7 ± 1.3</td>
<td>240.7 ± 32.5</td>
</tr>
<tr>
<td>END</td>
<td>12</td>
<td>-14.0 ± 1.6</td>
<td>26.6 ± 3.4</td>
</tr>
<tr>
<td>Inhibitor Combination</td>
<td>8</td>
<td>-11.0 ± 3.6</td>
<td>24.8 ± 7.1</td>
</tr>
<tr>
<td>Zero Na$^+e$</td>
<td>16</td>
<td>-9.4 ± 2.0</td>
<td>38.3 ± 5.2</td>
</tr>
<tr>
<td>Zero Na$^+e$, zero Ca$^{2+}e$</td>
<td>9</td>
<td>-51.1 ± 3.5</td>
<td>263.9 ± 45.9</td>
</tr>
<tr>
<td>ZeroCa$^{2+}e$</td>
<td>11</td>
<td>-49.6 ± 3.4</td>
<td>257.4 ± 39.7</td>
</tr>
<tr>
<td>100 µM GdCl$_3$</td>
<td>14</td>
<td>-52.4 ± 3.9</td>
<td>246.3 ± 41.3</td>
</tr>
<tr>
<td>500 µM ZnCl$_2$</td>
<td>11</td>
<td>-51.2 ± 2.7</td>
<td>235.7 ± 32.5</td>
</tr>
<tr>
<td>0.5 mM CaCl$_2$</td>
<td>9</td>
<td>-25.9 ± 4.6</td>
<td>59.1 ± 6.9</td>
</tr>
<tr>
<td>10 mM CaCl$_2$</td>
<td>12</td>
<td>-1.5 ± 2.4</td>
<td>22.9 ± 1.7</td>
</tr>
</tbody>
</table>
Fig. 4

Fura-2 imaging

Control

A

Cell-death assay

Control

B

END

END

C

END + 0 mM Ca^{2+}

END + 100 \mu M Gd^{3+}