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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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 it 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 used 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, thereby accounting for the calcium paradox. This injury-induced calcium-permeable channel represents a major source for the initial calcium entry following stroke, and it offers a new target for extending the therapeutic window for preventing neuronal death after the initial excitotoxic (stroke) injury.

Stroke is a leading cause of disability and death, yet its successful treatment is limited (Bonita et al., 2004). Glutamate is the predominant excitatory neurotransmitter in the mammalian central nervous system, and it is required for neural development, synaptogenesis, and alterations in synaptic plasticity (Dingledine et al., 1999). In excessive quantities, glutamate is thought to cause the neuronal damage observed following stroke, epilepsy, and traumatic brain injury (Siesjö and Bengtsson, 1989; Delorenzo et al., 2005). Protracted elevation in intracellular calcium $[\text{Ca}^{2+}]_i$ 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 dis-
turberance in neuronal Ca\textsuperscript{2+} homeostasis, producing pro-
trated elevations in [Ca\textsuperscript{2+}], that persist following the
removal of glutamate (Glau et al., 1990; Michaels and
Rothman, 1990; Dubinsky, 1993; Choi, 1995; Limbrick et al.,
1995, 2001, 2003). In addition, excitotoxic glutamate expos-
ure causes extended neuronal depolarization (END), a con-
dition that lasts for several hours after glutamate removal
(Sombati et al., 1991; Coulter et al., 1992; Limbrick et al.,
2003). Both the protracted [Ca\textsuperscript{2+}], elevations and END follow
similar time courses, require NMDA receptor activation and
an increase in [Ca\textsuperscript{2+}], for induction, and correlate with exci-
totoxic neuronal cell death (Sombati et al., 1991; Coulter et
al., 1992; Limbrick et al., 2003).

Conventional Ca\textsuperscript{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 oc-
curred, these Ca\textsuperscript{2+} entry antagonists are no longer effective
in blocking the Ca\textsuperscript{2+} entry and in reducing the elevated
[Ca\textsuperscript{2+}], (the Ca\textsuperscript{2+} plateau). Thus, traditional Ca\textsuperscript{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\textsuperscript{2+}
paradox of neuronal death in stroke, and they refer to the un-
expected finding that conventional Ca\textsuperscript{2+} entry antagonists
do not prevent Ca\textsuperscript{2+} entry or END after glutamate excito-
toxicity (Lee et al., 1999; Horn and Limburg, 2001; Ikonomidou
and Turski, 2002). Explaining the cause of the Ca\textsuperscript{2+} paradox
of neuronal death in stroke is one of the important problems
in neuroscience research, and it 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\textsuperscript{2+}
paradox identified that an influx of extracellular Ca\textsuperscript{2+}
was underlying the genesis of END. However, that study
could not identify the source or nature of this postinjury Ca\textsuperscript{2+}
entry (Limbrick et al., 2003). Therefore, it is important to ex-
plain this Ca\textsuperscript{2+} paradox and to understand the continued
Ca\textsuperscript{2+} entry after injury despite the use of known Ca\textsuperscript{2+}
entry inhibitors to develop novel and effective stroke and brain
injury therapeutic agents.

In this study, using a well characterized in vitro hippocam-
pal neuronal culture model of glutamate excitotoxicity/
stroke, we investigated the cause of the Ca\textsuperscript{2+} entry after
injury that is resistant to known Ca\textsuperscript{2+} entry inhibitors. Ex-
periments were directed at evaluating the development of an
injury induced Ca\textsuperscript{2+} current that underlies END and cell
death that is not blocked by conventional Ca\textsuperscript{2+} entry inhib-
itors. Studies were also directed at demonstrating that this
novel injury-induced Ca\textsuperscript{2+} current accounts for the Ca\textsuperscript{2+}
paradox, because traditional Ca\textsuperscript{2+} entry inhibitors, including
blockers for L-, N-, P/Q-, and T-type Ca\textsuperscript{2+} channels, NMDA/
AMPA/KCA channels, stretch-activated channels, and other
injury-induced cation channels, such as the TRPM-7 and
acid-sensing channels, did not block this Ca\textsuperscript{2+} entry. Fur-
thermore, our results also demonstrate that there is a ther-
apeutic window of opportunity of at least 1 h to block this
current, decrease the elevated [Ca\textsuperscript{2+}], reverse END, and
prevent neuronal death. The development of a novel injury-
induced Ca\textsuperscript{2+}-permeable channel provides a molecular basis
for the postinjury Ca\textsuperscript{2+} entry current responsible for produc-
ing END (Limbrick et al., 2003), and it explains why many of
the therapeutic trials using conventional strategies to inhibit
Ca\textsuperscript{2+} entry have not been effective in treating stroke. Acti-
vation of this injury-induced Ca\textsuperscript{2+}-permeable channel rep-
resents an early step in the cascade leading to excitotoxic
neuronal death, and it offers potential insight into developing
novel therapeutic interventions to prevent brain injury from
stroke.

Materials and Methods

Materials. All the reagents were purchased from Sigma-Aldrich
(St. Louis, MO), unless otherwise noted. α-Methyl-4-carboxyphenyl
glycine (mGCPG), SKF-96365, and tetrodotoxin (TTX) were obtained
from Tocris Cookson Inc. (Ballwin, MO), Calbiochem (San Diego,
CA), and Alomone Labs (Jerusalem, Israel), respectively. 4,4'-Di-
thiooxanostilbene-2,2'-disulfonic acid (DIDS), N\textsuperscript{\textdagger}-nitro-
l-arginine methyl ester (L-NAME), and amiloride hydrochloride (AMISO)
were purchased from Sigma-Aldrich. Minimal essential medium,
L-glutamine, trypsin, penicillin-streptomycin, fetal bovine serum, and
horse serum used in the tissue culture preparation were obtained from
Invitrogen (Carlsbad, CA).

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 Vir-
ginia 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% CO\textsubscript{2},
95% air atmosphere, and used after 14 days in vitro.

Electrophysiology. Whole-cell patch-clamp analyses were per-
duced as described using an Axopatch 200A amplifier or an Axo-
patch 1D amplifier (Molecular Devices, Sunnyvale, CA) in voltage-
clamp mode (Hamill et al., 1981; Coulter et al., 1992). Membrane
potential was sequentially stepped from −90 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
four-pole Beasel filter (Frequency Devices, Haverhill, MA). The re-
cording solution contained 145 mM NaCl, 2.5 mM KCl, 10 mM
HEPES, 10 mM glucose, 2 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2} pH 7.3
(290 ± 10 mO\textsubscript{M}). The pipette solution contained 140 mM K\textsuperscript{+} glu-
conate, 10 mM HEPES, 1.1 mM EGTA, and 1 mM MgCl\textsubscript{2} pH 7.2
(290 ± 10 mO\textsubscript{M}). Depending upon the experiments, various inhib-
itors were included in the recording solution. In establishing the
whole-cell configuration, gigaseal formation was verified, pipette
capacitance was canceled, and gentle suction was applied. Cells that
required >3 applications of suction for whole-cell access were dis-
carded. Once whole-cell access was established, whole cell capaci-
tance was canceled. Series resistance was generally 4 to 9 M\textsubscript{Ω}, but it
was reduced by 75 to 80% using the compensation circuit of the
amplifier. Series resistance error was generally between 3 and 5 mV
(but always <9 mV).

Cell-attached single-channel recordings were performed as de-
scribed previously (Hamill et al., 1981). Fire-polished Sylgard
(Dow Corning, Midland, MI)-coated borosilicate glass pipettes had a resis-
tance of 7 to 10 M\textsubscript{Ω} when filled with recording solution containing
145 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 10 mM glucose, 2 mM
CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2} pH 7.3 (290 ± 10 mO\textsubscript{M}). Depending upon
the experiment, various inhibitors were included in the pipette sol-
sution. Neurons were bath-perfused with a "high-K\textsuperscript{+}" 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

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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 (5 μM nifedipine), NMDA channel (10 μM MK-801), AMPA/KA channels (10 μM CNQX), the TRP channel family, and stretch-activated ion channels (10 μM GdCl_3) (Sattler et al., 1998; Aarts et al., 2003). Recordings in the presence this Ca^{2+} entry inhibitor cocktail have 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 to provide 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 they were sensitive to K^+ channel blockers (4-aminopyridine and charybotoxin). In contrast, excitotoxic glutamate injured neurons in the presence of Ca^{2+} entry inhibitors mixture manifest END (Sombati et al., 1991; Couler 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 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_reversal) of approximately +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 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 GdCl_3 or removal of [Ca^{2+}]_i after glutamate excitotoxicity (Limbrick et al., 2003). To investigate whether 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 100 μM GdCl_3 or removal of [Ca^{2+}]_i in the postglutamate END period abolished the inward current and restored the I-V profile identical to that of a control neuron (Fig. 1, E and F). In addition, Gd^{3+}-treated neurons (n = 14) and neurons recorded in absence of [Ca^{2+}]_i (n = 11) had a mean membrane potential of −52.4 ± 3.9 and −49.6 ± 3.4 mV, respectively. The mean input resistance for these two

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>No. 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>0 [Na^+]_i</td>
<td>16</td>
<td>−9.4 ± 2.0</td>
<td>38.3 ± 5.2</td>
</tr>
<tr>
<td>0 [Na^+]_i, 0 [Ca^{2+}]_i</td>
<td>9</td>
<td>−51.1 ± 3.5</td>
<td>263.9 ± 45.9</td>
</tr>
<tr>
<td>0 [Ca^{2+}]_i</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>4</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>
conditions was 246.3 ± 41.3 and 257.4 ± 39.7 MΩ, respectively. 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 Ca²⁺ entry inhibitors, ruling out the involvement of voltage-dependent Ca²⁺ channels. Together, these results indicate that excitotoxic glutamate exposure produces END and activates a novel channel activity that carries IEIC in injured neurons.

**Calcium Ions Are the Primary Permeant Ions for IEIC and the IEIC Channel.** To confirm the ionic basis of END, IEIC, and the channel-mediating IEIC, major cations were sequentially replaced in the recording solution. Equimolar substitution of NaCl with NMDG failed to reverse END (Fig. 2A), they did not affect the magnitude of IEIC (Fig. 2B), and they 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), compared with END neurons (p = 0.101 and 0.093, respectively; n = 16; Table 1). These results indicate that Na⁺ influx did not significantly contribute to IEIC.

Removing [Ca²⁺]e following glutamate excitotoxicity completely reversed END (Fig. 2A), abolished IEIC (Fig. 2B), and also abolished single-channel events (Fig. 2C). Neurons subjected to Ca²⁺-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 values for control neurons (p = 0.075 and 0.745, respectively; n = 11; Table 1). Permeability ratios calculated from the Goldman-Hodgkin-Katz equation (Hille,
2001), using the steady-state $E_{\text{rev}}$, indicated that $I_{\text{EIC}}$ is mediated by a highly selective $\text{Ca}^{2+}$ conductance that is 50 times more permeable to $\text{Ca}^{2+}$ than $\text{Na}^{+}$ ($P_{\text{Ca}}/P_{\text{Na}} = 12.5:0.25$).

To further establish that $\text{Ca}^{2+}$ influx was responsible for END and that it was the major ion permeating the $I_{\text{EIC}}$ channel, we measured $[\text{Ca}^{2+}]_i$ using Fura-2 imaging. Substituting NaCl with NMDG had no effect on $[\text{Ca}^{2+}]_i$. Conversely, removing $[\text{Ca}^{2+}]_e$ reversed the elevated $[\text{Ca}^{2+}]_i$ observed after glutamate excitotoxicity (Fig. 2D).

The $\text{Ca}^{2+}$ dependence of $I_{\text{EIC}}$ was then evaluated by investigating the effects of variable $[\text{Ca}^{2+}]_e$ on magnitude of $I_{\text{EIC}}$. The net peak inward currents reduced from $-3932.3 \pm 635.5$ to $-1119.7 \pm 327.4$ pA at $-90$ mV during 2 and 0.5 mM $[\text{Ca}^{2+}]_e$ (Fig. 2E), and it also caused a significant shift in the $E_{\text{rev}}$ from $+40.5 \pm 2.2$ to $-20.6 \pm 2.0$ mV, respectively. This value agreed with the theoretical $E_{\text{rev}}$ value of approximately $-22$ mV determined by the Nernst equation for 0.5 mM $[\text{Ca}^{2+}]_e$, and it resulted in the predicted linear relationship of $E_{\text{rev}}$ versus log$_{10} [\text{Ca}^{2+}]_e$ (Fig. 2F). The membrane potential for neurons in 0.5 mM CaCl$_2$ was $-25.9 \pm 4.6$ mV, and the input resistance was $59.1 \pm 6.9$ MΩ. These values represent significant changes from those measured in 2 mM CaCl$_2$ for END ($p = 0.013$ and $<0.001$, respectively; $n = 9$; Table 1).

Conversely, increasing $[\text{Ca}^{2+}]_e$ from 2 to 10 mM resulted in an increased net peak inward current of $-5500.8 \pm 457.8$ pA (Fig. 2E), and it resulted in a membrane potential of $-1.5 \pm 2.4$ mV and an input resistance of $22.9 \pm 1.7$ MΩ. Increasing $[\text{Ca}^{2+}]_e$ to 10 mM caused a shift in the $E_{\text{rev}}$ from $+40.5 \pm$...
2.2 to $+50.1 \pm 4.2$ mV. The observed shift of $E_{\text{mem}}$ with 10 mM CaCl$_2$ was slightly less than the predicted $E_{\text{mem}}$ (approximately $+62$ mV), and it may be due to saturation of $E_{\text{mem}}$ when plotted against $\log_{10} [\text{Ca}^{2+}]_i$ (Fig. 2F). This observation is consistent with the concept of permeant ion rectification, where saturation of $I_{\text{KIC}}$ occurs with respect to $[\text{Ca}^{2+}]_i$ (Hille, 2001). Thus, $I_{\text{KIC}}$ followed Nernstian predictions in response to changes in $[\text{Ca}^{2+}]_i$.

Taken together, the following evidence supports the conclusion that the injury-induced Ca$^{2+}$-permeable channel is responsible for carrying the $I_{\text{KIC}}$ that mediates END: 1) activation of the ion channel in the postglutamate END period coincides with the appearance of $I_{\text{KIC}}$; 2) END, $I_{\text{KIC}}$, and the Ca$^{2+}$-permeable channel demonstrate identical ionic selectivity and are abolished in the absence of $[\text{Ca}^{2+}]_i$; 3) END, $I_{\text{KIC}}$, and the Ca$^{2+}$-permeable channel all manifest identical insensitivity to blockade by Ca$^{2+}$ and CNQX, was used to wash out glutamate. Because alterations in sensitivity to blockade by Ca$^{2+}$ and Gd$^{3+}$ 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 $I_{\text{KIC}}$.** We used extensive pharmacological studies to establish that the $I_{\text{KIC}}$-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 (VGCCs) 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. To investigate contribution of VGCCs to END, a combination of effective concentrations of L-type VGCC antagonist (5 $\mu$M nifedipine), N-type VGCC antagonist (1 $\mu$M $\alpha$-conotoxin GVIA), P/Q-type VGCC antagonist (100 nM $\Omega$-conotoxin MVIIC), or T-type VGCC antagonist (1 mM ethosuximide), along with other components of Ca$^{2+}$ entry inhibitor mixture (such as MK-801 and CNQX), was used to wash out glutamate. Because alterations in VGCC gating could also occur during or after excitotoxic glutamate exposure, we included 200 $\mu$M 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 $\mu$M (Caldwell et al., 1998). However, this concentration did not inhibit END or decrease $I_{\text{KIC}}$ or single-channel activity after glutamate excitotoxicity. Thus, although use of 10 $\mu$M GdCl$_3$ inhibited these other channels under our conditions, 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 $\mu$M. This is a very large difference in concentration, and it 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 a prolonged period in the depolarized END phase. Moreover, VGCCs 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 $\mu$M) and APV (50 $\mu$M) 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 noncompetitive (MK-801) pharmacological inhibitors to NMDA channel do not block the postglutamate 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. Likewise, the inability of AMPA/KA receptor antagonists (10 $\mu$M CNQX, 10 $\mu$M NBQX) or the metabotropic glutamate receptor antagonist (250 $\mu$M mCPG) used 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 VGCCs. However, Zn$^{2+}$ at a lower 5 $\mu$M 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 postglutamate membrane potential (Fig. 3A). Conversely, inclusion of 500 $\mu$M ZnCl$_2$ 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 (50 $\mu$M bepridil or [Na$^+$]$_i$, removal), chloride channels (100 $\mu$M DIDS), and stretch receptors channels (10 $\mu$M GdCl$_3$) also had no effect on diminishing the END potentials after glutamate excitotoxicity (Fig. 3A). In addition, the strict ionic selectivity, the differential permeability of $I_{\text{KIC}}$ and the fact that changes in $[\text{Ca}^{2+}]_i$ could be measured throughout our experiments demonstrated that $I_{\text{KIC}}$ was not the result of a nonspecific 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 (10 $\mu$M SKF-96365), ryanodine receptors (20 $\mu$M dantrolene), or inositol trisphosphate receptors (2 mg/ml heparin) or intracellular Ca$^{2+}$ release inhibitor (1 $\mu$M thapsigargin) (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 phosphocreatine) or by inhibiting mitochondrial Ca$^{2+}$ release (10 $\mu$M rhodamine; data not shown) also had no effect on reducing END (Fig. 3A). Indeed, mitochondria maintain their resting level for Ca$^{2+}$ for about 45 min 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 \([\text{Ca}^{2+}]_{i}\) accumulation, our results demonstrate that \(I_{\text{IEIC}}\) is responsible for majority of the early Ca\(^{2+}\) influx after glutamate excitotoxicity.

**Neuroprotection with Gadolinium: Evidence That \(I_{\text{IEIC}}\) Accounts for the Ca\(^{2+}\) Paradox.** To test whether activation of \(I_{\text{IEIC}}\) could explain the Ca\(^{2+}\) paradox of excitotoxic neuronal death, we investigated whether blocking this channel could prevent neuronal death after glutamate excitotoxicity. Treatment with 100 \(\mu\text{M}\) Gd\(^{3+}\) produced a significant reduction in the number of Annexin-positive cells (Figs. 3C and 4), and it also conferred neuroprotection when administered 1 h after excitotoxic injury (Fig. 3D). Furthermore, 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 neuronal NOS 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 (Vigé et al., 1993). Thus, addition of 100 \(\mu\text{M} N^\omega\)-nitro-L-arginine to the medium either 5 min before and during 500 \(\mu\text{M}\) L-glutamate exposure for 5 min decreased the amino acid-induced neurotoxicity only by 20%. However, when added 5 min before L-glutamate and kept in contact with neurons for the following 24 h after glutamate removal, 100 \(\mu\text{M} N^\omega\)-nitro-L-arginine antagonized the glutamate-induced neurotoxicity by more than 70% (Vigé et al., 1993). Several research groups have reported that inhibition of NOS following a 5- to 10-min exposure to 300 to 500 \(\mu\text{M}\) NMDA did not attenuate neuronal death in primary
cortical cultures from rat and mouse (e.g., 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 (e.g., 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 \([\text{Ca}^{2+}]_i\) to basal levels for up to 1 h after removal of \([\text{Ca}^{2+}]_i\) (Figs. 3E and 4). However, beyond this time point, the percentage of neurons that could restore basal \([\text{Ca}^{2+}]_i\) levels started to decrease despite absence of \([\text{Ca}^{2+}]_i\). 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 \([\text{Ca}^{2+}]_i\), while the I_{EIC} channel is the major source of elevated \([\text{Ca}^{2+}]_i\). In addition, as shown in Fig. 1A, injured neurons revealed I_{EIC} despite the presence of \([\text{Ca}^{2+}]_i\) entry inhibitors. Thus, these data provide an explanation for the \([\text{Ca}^{2+}]_i\) paradox by demonstrating that blocking I_{EIC}-Ca^{2+}-permeable channel was able to prevent \([\text{Ca}^{2+}]_i\) entry and inhibitor-resistant \([\text{Ca}^{2+}]_i\) entry and reverse END, and inhibit cell death.

**Comparison of I_{EIC} to Other Injury-Induced Cation Currents.** It is important to compare I_{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_{OOGD}) in cortical neurons (Aarts et al., 2003). Two hours of anoxia induced I_{OOGD}, which was inhibited by 300 \(\mu\)M L-NAME or 10 \(\mu\)M GdCl\(_3\) and increased in magnitude upon \([\text{Ca}^{2+}]_i\) removal (Aarts et al., 2003). In contrast, induction of I_{EIC} was rapid, and treatments with L-NAME or 10 \(\mu\)M GdCl\(_3\) during and after glutamate excitotoxicity failed to reverse END (Fig. 3A), inhibit \([\text{Ca}^{2+}]_i\) influx (data not shown), or prevent neuronal death (Fig. 3C). Moreover, I_{EIC} decreased in magnitude upon \([\text{Ca}^{2+}]_i\) removal. Thus, based on the kinetic characteristics and pharmacological comparisons, it seems that TRPM-7 channels are not responsible for mediating I_{EIC}.

Acidosis from ischemia induces the activation of amiloride-sensitive high Na\(^+\)-low Ca\(^{2+}\)-permeable cation channels.

![Fura-2 imaging and Cell-death assay](image-url)
(ASICs) (Xiong et al., 2004). Amiloride (100 μM) or its derivative, bepridil (50 μM), had no significant effect on END (Fig. 3A), they failed to block Ca\(^{2+}\) influx (data not shown), and they did not prevent cell death after glutamate excitotoxicity (Fig. 3C). Furthermore, the activation of ASICs was unlikely because our perfusion conditions prevented the development of acidic conditions, demonstrating that ASICs are not responsible for mediating IEIC.

Excitotoxic injury with NMDA can also induce a postexposure current (I_{pe}) that was shown to be not selective for Ca\(^{2+}\) (P_{Ca}:P_{Na} = 7:1) and not altered by removal of [Ca\(^{2+}\)]_o in acutely isolated hippocampal neurons (Chen et al., 1997). In contrast, steady-state I_{EIC} has a high Ca\(^{2+}\) selectivity (P_{Ca}:P_{Na} = 50:1), and it is abolished upon omission of [Ca\(^{2+}\)]_o, demonstrating that I_{pe} is not responsible for mediating I_{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 postglutamate treatment paradigm and that I_{EIC} is a unique Ca\(^{2+}\) current that 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_{EIC}), primarily responsible for the initial sustained increases in [Ca\(^{2+}\)]_i, following neuronal injury that maintains END and ultimately leads to neuronal death. Inhibiting this I_{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_{EIC}-Ca\(^{2+}\)-permeable channel following glutamate excitotoxicity provides the first molecular insight into the Ca\(^{2+}\) paradox, and it explains why many of the clinical trials using 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 (Kristián and Siesjö, 1998). Thus, a critical question relates to the source of this Ca\(^{2+}\). Whereas 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_{EIC}-Ca\(^{2+}\)-permeable channel is responsible for the majority of the Ca\(^{2+}\) influx during the 1st hour after glutamate excitotoxicity. During this 1-h time window, intervention with Gd\(^{3+}\) or removal of [Ca\(^{2+}\)]_o can restore elevated [Ca\(^{2+}\)]. However, beyond this time point, in addition to I_{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\(^{2+}\)], despite removal of [Ca\(^{2+}\)].

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\(^{2+}\)]_o with [Ba\(^{2+}\)]_o during glutamate excitotoxicity both caused neuronal depolarization, but they 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.

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\(^{2+}\)]_i represent a prolonged imbalance in Ca\(^{2+}\) homeostasis, and they correlate with subsequent excitotoxic neuronal death (Dubinsky, 1993; Limbrick et al., 1995). This [Ca\(^{2+}\)]_i deregulation could result either from a persistent influx of [Ca\(^{2+}\)]_o and/or from sustained impairment of neuronal Ca\(^{2+}\) sequestration/extrusion mechanisms. Indeed, recent discoveries that TRPM-7 (Aarts et al., 2003) and ASICs (Xiong et al., 2004) allow for Ca\(^{2+}\) entry during 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\(^{2+}\)]_i elevations following glutamate excitotoxicity.

Earlier attempts to elucidate the basis of the postexcitotoxic injury-induced Ca\(^{2+}\) paradox indicated that an influx of extracellular Ca\(^{2+}\) was responsible for END (Limbrick et al., 2003). However that study did not identify the source or nature of this postinjury Ca\(^{2+}\) entry. The data presented here are a major advance over this earlier work, and they demonstrate 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 study 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 acti-
vated by glutamate excitotoxicity and blocking its activity after the excitotoxic insult prevents [Ca2+]i accumulation and neuronal cell death. These findings suggest that activation of I_{KCa}-Ca2+-permeable channel could represent an early step in the genesis of the injury induced [Ca2+]i 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. Elicitation of the I_{KCa} may provide a new target for a significant extension of the therapeutic window to prevent neuronal death in stroke and offer new hope in the search for novel agents to treat stroke and excitotoxic brain injury.

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References


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