Hydrogen Peroxide Potentiates Volume-sensitive Excitatory Amino Acid Release via a Mechanism Involving Ca<sup>2+</sup>/Calmodulin-dependent Protein Kinase II*<sup>¶,§</sup>

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Excessive excitatory amino acid (EAA) release in cerebral ischemia is a major mechanism responsible for neuronal damage and death. A substantial fraction of ischemic EAA release occurs via volume-regulated anion channels (VRACs). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which is abundantly produced during ischemia and reperfusion, activates a number of protein kinases critical for VRAC functioning and has recently been reported to activate VRACs. In the present study, we explored the effects of H<sub>2</sub>O<sub>2</sub> on volume-dependent EAA release in cultured astrocytes, measured as the release of preloaded <sup>3</sup>H]-glutamate. 100–1,000 μM H<sub>2</sub>O<sub>2</sub> enhanced swelling-induced EAA release by ~2.5-3-fold (EC<sub>50</sub> ~ 10 μM). The VRAC blockers ATP, phloretin, and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) potently inhibited both control swelling-induced and the H<sub>2</sub>O<sub>2</sub>-potentiated release, suggesting a role for VRACs. The H<sub>2</sub>O<sub>2</sub>-induced component of EAA release was attenuated by the Ca<sup>2+</sup>-chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxyethyl ester (BAPTA-AM) and completely eliminated by the calmodulin antagonists trifluoperazine and W-7 and the Ca<sup>2+</sup>-/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93. Inhibitors of tyrosine kinases, protein kinase C, and the myosin light chain kinase were ineffective in blocking the H<sub>2</sub>O<sub>2</sub> response. H<sub>2</sub>O<sub>2</sub> treatment of swollen astrocytes, but not swelling alone, resulted in CaMKII activation that was inhibited by KN-93, as determined by a phospho-Thr286 CaMKII antibody. These data demonstrate that H<sub>2</sub>O<sub>2</sub> strongly up-regulates astrocytic volume-sensitive EAA release via a CaMKII-dependent mechanism and in this way may potently promote pathological EAA release and brain damage in ischemia.

The pathological release of the excitatory amino acids (EAs)<sup>1</sup> glutamate and aspartate, followed by the subsequent activation of glutamate receptors, is considered an early obligatory event in the excitotoxicity cascade that leads to neuronal damage and death in cerebral ischemia (1, 2). Volume-regulated anion channels (VRACs) are thought to constitute a major pathway for EAA release, as suggested by the inhibition of pathological glutamate and aspartate release by several VRAC blockers in animal models of global and focal cerebral ischemia (3–6). VRACs are ubiquitously expressed, activated in response to cell swelling, and characterized by Eisenman type I anion selectivity (I<sup>−</sup> > NO<sub>3</sub> > Br < CI > F <sup>−</sup> ), moderate outward rectification, and inactivation at positive potentials (7–9). Although the molecular identity of VRACs and the precise mechanisms of their regulation are not known, VRAC activation is probably provoked by pronounced pathological cell swelling, primarily seen in astrocytes in ischemia (12–14).

It is not known whether, in addition to cell swelling, other pathological factors play a role in VRAC-mediated EAA release. Several recent publications have demonstrated that hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) induces typical VRAC currents in the absence of cell swelling in HeLa and hepatoma cell lines (15, 16) and positively regulates swelling-activated organic osmolyte release in NIH3T3 fibroblasts (17). Because H<sub>2</sub>O<sub>2</sub> and other reactive oxygen species are abundantly produced in ischemia and during reperfusion (18, 19), it is plausible that H<sub>2</sub>O<sub>2</sub> causes the activation or positive modulation of excitatory amino acid release in the ischemic brain. In line with such a suggestion, reactive oxygen species positively modulate the activity of a number of protein kinases, such as tyrosine kinases, mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) (20–22). All of these kinases have been found to contribute to VRAC activation and/or modulation in a variety of cell types (9, 23–28). Robust changes in tyrosine phosphorylation and MAPK activity, as well as the translocation of PKC and CaMKII from the cytosol to the membrane fraction, have been found in ischemia (29–32). In the present work, we examined the potential role of H<sub>2</sub>O<sub>2</sub> in mediating the activation or modulation of excitatory amino acid release via a putative VRAC pathway and explored intracellular signaling mechanisms involved in the H<sub>2</sub>O<sub>2</sub> effects in primary astrocyte cultures.

EXPERIMENTAL PROCEDURES

Chemicals—The neutral protease dispase was obtained from Roche Diagnostics, and all other cell culture reagents were from Invitrogen.

PTA, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; AM, acetoxymethyl ester; MLCK, myosin light chain kinase.
**RESULTS**

**Hydrogen Peroxide Potentiates Swelling-sensitive EAA Release**—We used d-[3H]aspartate as a tracer for EAA because it is not metabolized and is released through the same swelling-induced permeability pathways as L-[3H]glutamate (36). In astrocyte cultures, cell swelling induced by a 30% reduction in medium osmolarity (~90 mOsm) initiated a large release of preloaded d-[3H]aspartate (Fig. 1A). H$_2$O$_2$, given 10 min before and continuously during hypo-osmotic medium application, potentiated peak swelling-induced EAA release in a dose-dependent manner (Fig. 1B). The H$_2$O$_2$-effect was saturated at concentrations ≥100 μM (EC$_{50}$ = 8.2 ± 4.0 μM, as determined by a dose-response curve fit, R$^2$ = 0.98). 500 μM H$_2$O$_2$ was used throughout the study to ensure maximal activity. 100–1,000 μM H$_2$O$_2$ enhanced swelling-induced EAA release by ~2.5–3-fold (Fig. 1, A and B). In the absence of cell swelling, 500 μM H$_2$O$_2$ failed to initiate any rapid increases in EAA release, although it did induce a gradual upward shift in d-[3H]aspartate baseline values (Fig. 1A). After removal of H$_2$O$_2$, we typically observed a secondary increase in d-[3H]aspartate release compared with control baseline release, the degree of which varied between cell culture preparations. Because this secondary release was observed only after the removal of H$_2$O$_2$, we did not investigate its origin. To verify that the H$_2$O$_2$-induced potentiation of EAA release was not caused by additional cell swelling, we monitored relative cell volume changes using calcine fluorescence (35). Exposure to hypo-osmotic medium produced a ~4.5% increase in calcine fluorescence (Fig. 1C), which positively correlates to changes in cell volume (35). 500 μM H$_2$O$_2$ produced no additional changes in calcine fluorescence before and during hypo-osmotic medium exposure (Fig. 1C).
500 μM H$_2$O$_2$ produced no changes in astrocyte morphology (Supplemental Figure). To establish that the H$_2$O$_2$ enhancement of hypo-osmotic EAA release is mediated by VRACs, we used several VRAC blockers and compared their effects on EAA release in the presence or absence of H$_2$O$_2$. 10 mM ATP (which at this concentration acts as an open pore VRAC blocker (8)), 100 μM phloretin (which inhibits VRACs but not cystic fibrosis transmembrane conductance regulator channel or Ca$^{2+}$-activated Cl$^-$ channels at the concentration used (38)), and the broad spectrum anion channel blocker 100 μM NPPB (8) all potently inhibited hypo-osmotic medium-induced EAA release both during control astrocyte swelling and in the presence of 500 μM H$_2$O$_2$ (Fig. 2B).

Intracellular Signaling Mechanisms Involved in the H$_2$O$_2$ Effect on EAA Release—H$_2$O$_2$ activates non-receptor tyrosine kinases of the src family, receptor tyrosine kinases, and, downstream, enzymes of the MAPK cascade (22, 39), all of which have been found to contribute to VRAC activation (23–25). Therefore, we tested for the contribution of tyrosine and MAP kinases in mediating the H$_2$O$_2$ effect on swelling-induced EAA release. The selective src kinase inhibitors PP2 (10 μM) or SU6656 (5 μM) (40), the receptor tyrosine kinase inhibitor tyrphostin A51 (20 μM) (41), or the MEK inhibitor U0126 (10 μM) (42), did not block the H$_2$O$_2$-induced potentiation of EAA release (Fig. 3A).

We also examined the potential role of PKC because this enzyme is also activated by H$_2$O$_2$ (43, 44) and has been shown to regulate VRAC activity (26, 45). The potent PKC inhibitors 1 μM Go6983, 1 μM bisindolylmaleimide I, and 5 μM Ro32-0432 (46–48) were ineffective in blocking the H$_2$O$_2$-enhanced EAA release during cell swelling (Fig. 3B). In contrast, the PKC inhibitor 5 μM chelerythrine (52) completely blocked the H$_2$O$_2$-evoked enhancement in EAA release (Fig. 3B).

Two Ca$^{2+}$/calmodulin-dependent enzymes known to be activated by H$_2$O$_2$, the myosin light chain kinase (MLCK), and CaMKII, have also been found to regulate VRACs (20, 27, 49, 50). We therefore explored whether changes in intracellular Ca$^{2+}$ and the activation of Ca$^{2+}$/calmodulin-dependent enzymes are involved in the H$_2$O$_2$ effect. Using the fluorescent Ca$^{2+}$ indicator fluo-4-AM, we found that H$_2$O$_2$ pretreatment produces a steady rise in [Ca$^{2+}$], levels before the induction of cell swelling (Fig. 4, A and B). Hypo-osmotic medium-induced cell swelling itself caused a steep increase in [Ca$^{2+}$], followed by a gradual recovery toward baseline values (Fig. 4B). In the presence of 500 μM H$_2$O$_2$, swelling-induced [Ca$^{2+}$], release on average tended to be somewhat higher, but this effect was not statistically significant (Fig. 4B).

To test for Ca$^{2+}$ involvement in the H$_2$O$_2$ effect on EAA release, we pretreated cultured astrocytes for 20 min with the cell membrane-permeable Ca$^{2+}$ chelator 10 μM BAPTA-AM, followed by a 10-min wash out. BAPTA-AM pretreatment partially reduced the H$_2$O$_2$-induced increment in EAA release but had no statistically significant effect on control swelling-induced EAA release (Fig. 4C). The calmodulin antagonists trifluoperazine (20 μM) and W-7 (20 μM) (51, 52) completely eliminated the H$_2$O$_2$-induced potentiation of swelling-induced D-[3H]aspartate release (Fig. 4D). On the other hand, the inactive structural analogue of W-7, W-5 (20 μM), was ineffective in reducing the H$_2$O$_2$ effect (Fig. 4D). In contrast to their effects in the presence of H$_2$O$_2$, trifluoperazine and W-7 did not reduce EAA release under control hypo-osmotic conditions (Fig. 4D).

To determine the specific Ca$^{2+}$/calmodulin-dependent enzymes involved in the H$_2$O$_2$ effect, we used inhibitors of MLCK (ML-7 (40, 53)), and CaMKII (KN-93 (54)). Addition of 5 μM ML-7 during 500 μM H$_2$O$_2$ perfusion had no effect on the ability of H$_2$O$_2$ to enhance swelling-sensitive D-[3H]aspartate release (n = 4 per group, p = 0.243; data not shown). In contrast, the application of the CaMKII inhibitor 5 μM KN-93 completely abolished the effect of H$_2$O$_2$ but had no effect on control swelling-induced EAA release (Fig. 5A).

We further tested the effects of H$_2$O$_2$ on CaMKII activity using a phospho- Thr286 CaMKII antibody recognizing the autophosphorylated active form of CaMKII (55). In astrocyte lysates, this antibody recognized a single protein band of ~52 kDa that corresponds to the predicted molecular mass of CaMKII. CaMKII expression and loading levels were not different between treatment groups, as verified using a pan CaMKII antibody. We found that H$_2$O$_2$ caused CaMKII activation in swollen astrocytes, which was blocked by 5 μM KN-93 (Fig. 5, B and C). Hypo-osmotic medium-induced swelling itself did not increase CaMKII activity above basal levels (Fig. 5, B and C).

**DISCUSSION**

In the present study, we have found that hydrogen peroxide acts as a potent positive modulator of swelling-activated excitatory amino acid release in cultured astrocytes. When combined with hypo-osmotic swelling, H$_2$O$_2$ dose-dependently po-
tentiated swelling-induced D-[3H]aspartate release by up to 3-fold (EC50/H11015/10/H9262M). We found no morphological changes in astrocytes treated with 500/H9262M H2O2, which is in line with data showing that astrocytes are highly resistant to oxidative stress (56, 57). Furthermore, potentiation of swelling-induced D-[3H]aspartate release by H2O2 was not related to nonspecific changes in membrane permeability, monitored as the release of preloaded [51Cr]. H2O2 also did not enhance astrocyte swelling under hypo-osmotic conditions. Pharmacological analysis suggested that the H2O2-induced increase in EAA release is probably caused by the potentiation of VRAC activity in swollen cells. In our experiments, the VRAC blockers ATP (10 mM), phloretin (100 μM), and NPPB (100 μM) (8, 38) potently reduced EAA release in both the presence and absence of H2O2. Although there are currently no specific VRAC blockers available, the use of these three distinct inhibitors probably rules out alternative transport pathways that might also contribute to astrocytic EAA release. Such pathways might include other anion channels, a vesicular glutamate release pathway, connexin hemichannels, P2X7 receptor channels, and excitatory amino acid transporters working in the reverse mode (58–63).

VRACs, which are typically activated in response to cell swelling, are ubiquitously expressed and permeable toward...
small organic molecules in astrocytes and other cell types (10, 36, 64, 65). Two recent publications have shown that H₂O₂ induces a gradual increase in Cl⁻/H⁺ conductance in non-swollen cells, which exhibits the typical current/voltage relationship and pharmacological properties of VRACs (15, 16). Although H₂O₂ strongly up-regulated volume-sensitive EAA release in our experiments, in the absence of cell swelling, even 20-min H₂O₂ treatment produced only a slight upward shift in the baseline release rate that was more pronounced after the removal of H₂O₂. Such a difference between our efflux experiments and the studies showing H₂O₂-induced Cl⁻/H⁺ current activation in non-swollen cells may be related to cell type differences or a modified intracellular milieu resulting from cell dialysis in electrophysiological experiments. Lambert (17) has found that H₂O₂ induces the positive modulation of taurine release only in swollen NIH 3T3 fibroblasts.

Although H₂O₂-dependent VRAC activation and modulation of organic osmolyte release has been described in several cell lines (15–17), the intracellular signaling events responsible for such effects have not been determined. It is well known that H₂O₂ stimulates numerous intracellular signaling pathways, including tyrosine kinases, MAPKs, PKC, MLCK, and CaMKII, and inhibits tyrosine phosphatases (20–22, 49, 66). Because the same protein kinases have also been found to regulate VRAC activity, we hypothesized that H₂O₂ exerts its actions on astrocytic amino acid release via alterations in VRAC-associated intracellular signaling pathways. In our previous work, we found that the reactive nitrogen species peroxynitrite potentiates swelling-activated D-[³H]aspartate release via a tyrosine kinase-dependent mechanism (67). Therefore, we tested for the involvement of tyrosine kinases but found that none of the inhibitors of non-receptor tyrosine kinases (PP2 and SU6656) or receptor tyrosine kinases (tyrphostin A51) or the MEK inhibitor (U0126) prevented the H₂O₂ effect. PKC was also a potential candidate, in that it has been found to be activated by H₂O₂ (43, 44) and is also critical for the activation
or modulation of VRACs (26, 45) and volume-dependent organic osmolyte release (50). However, we found that several potent PKC inhibitors (Go6983, bisindolylmaleimide I, and Ro32-0432) were ineffective in attenuating the H2O2-response. The PKC inhibitor chelerythrine blocked the H2O2-effect, but because the other PKC blockers did not replicate this effect, it is unlikely to be related to PKC inhibition.

H2O2 also elevates [Ca2+]i in a variety of cell lines (22, 68). Therefore, we investigated the possible involvement of Ca2+ and calmodulin in mediating the H2O2 effect on amino acid release. Because cell swelling itself is known to stimulate [Ca2+]i, increases in a majority of cell types, including cultured astrocytes (69), we sought to determine whether the presence of H2O2 during astrocytic swelling would further potentiate the [Ca2+]i signal. In our experiments, H2O2 initiated a significant gradual [Ca2+]i rise before the onset of hypo-osmotic swelling. Under hypo-osmotic conditions, H2O2 modestly potentiated swelling-induced [Ca2+]i increases, but this effect was not statistically significant. Nonetheless, the Ca2+-chelator BAPTA-AM partially blocked the H2O2-induced EAA release. In line with the BAPTA-AM data, the calmodulin antagonists trifluoperazine and W-7 completely eliminated the H2O2 effect. Partial inhibition of the H2O2-induced potentiation of EAA release by 10 μM BAPTA-AM, compared with full inhibition by the calmodulin antagonists, may be caused by insufficient intracellular Ca2+ chelation. We attempted to use a higher BAPTA-AM concentration, but it caused potent nonspecific EAA release on its own. In contrast to their effects on the H2O2-response, BAPTA-AM and the calmodulin blockers did not significantly affect control swelling-induced 3H-aspartate release. These observations are consistent with a number of publications indicating that although swelling-induced [Ca2+]i increases are not necessary for VRAC activation, additional elevations in [Ca2+]i by the Ca2+-ionophore ionomycin and [Ca2+]i-releasing agonists potently modulate VRAC activity (27, 70–73).

To establish the Ca2+/calmodulin-dependent mechanism responsible for the H2O2 effect, we looked into the involvement of two Ca2+/calmodulin-dependent enzymes, MLCK and CaMII. The MLCK inhibitor ML-7 was ineffective in blocking the H2O2-response, ruling out MLCK involvement. Although MLCK is a potent positive modulator of VRAC activity in endothelial cells (74), in astrocytes and other cell types, this Ca2+/calmodulin-dependent enzyme seems not to contribute to the activation of VRACs and volume-dependent organic osmolyte release (75, 76).

In contrast to ML-7, the CaMII inhibitor KN-93 completely eliminated the H2O2 effect without altering control volume-sensitive EAA release. These data suggest that CaMII is a critical component of the H2O2-dependent potentiation of swelling-sensitive EAA release. Similar to our findings, Cardin et al. have demonstrated that the Ca2+-ionophore ionomycin enhances volume-sensitive taurine release in cerebellar astrocyte cultures through a CaMII-dependent mechanism (27). In the same study, CaMII inhibition was ineffective in blocking control swelling-induced release in the absence of ionomycin (27).

Using a phospho-Thr286 antibody recognizing the autophosphorylated active form of CaMII (55), we confirmed that H2O2 potentiates swelling-activated excitatory amino acid release via a CaMII-dependent mechanism (27). In the presence of the CaMII inhibitor KN-93, the treatment conditions were similar to those shown in A. Astrocytes were lysed at the time point corresponding to the 25th minute of the efflux experiments. B shows representative Western blots for phospho-Thr286 CaMII and pan-CaMII antibodies. Phosphorylation levels were compared with cells kept in basal medium for 25 min. The average optical densities ± S.E. of the immunoreactive bands from five different cell preparations are presented in C. *p < 0.05, H2O2 versus all other groups.

VRACs probably play a major role in mediating excessive EAA release during cerebral ischemia (5–8). The potent VRAC blocker tamoxifen reduces infarct size after transient and global ischemia, although the exact mechanism of tamoxifen protection remains to be elucidated (79, 80). Astrocytes are a plausible site for VRAC activation because, unlike neurons, they are highly susceptible to ischemia-induced cell swelling (12–14). However, swelling may not be the only factor responsible for pathological VRAC regulation. The massive release of neurotransmitters and neuromodulators from depolarized and damaged neuronal cells, along with the excessive production of reactive nitrogen and oxygen species, causes alterations in intracellular signaling (18, 22). In particular, severalfold increases in tyrosine phosphorylation and MAPK activity, as well as the robust translocation of PKC and CaMII from the cytosol to the membrane fraction, have been found in animal ischemia models (29–32). Our in vitro data demonstrate that H2O2 strongly up-regulates astrocytic VRAC activity via a CaMII-dependent mechanism and in this way may potentially

![Graph](http://www.jbc.org/Downloaded from)
promote pathological excitatory amino acid release in the ischemic brain.

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