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Oxygen/Glucose Deprivation Induces a Reduction in Synaptic AMPA Receptors on Hippocampal CA3 Neurons Mediated by mGluR1 and Adenosine A3 Receptors

Siobhan H. Dennis, Nadia Jaafari, Helena Cimarosti, Jonathan G. Hanley, Jeremy M. Henley, and Jack R. Mellor

Medical Research Council Centre for Synaptic Plasticity, School of Physiology and Pharmacology and School of Biochemistry, University of Bristol, Bristol BS8 1TD, United Kingdom, and School of Pharmacy, University of Reading, Reading RG6 6UB, United Kingdom

Hippocampal CA1 pyramidal neurons are highly sensitive to ischemic damage, whereas neighboring CA3 pyramidal neurons are less susceptible. It is proposed that switching of AMPA receptor (AMPAR) subunits on CA1 neurons during an in vitro model of ischemia, oxygen/glucose deprivation (OGD), leads to an enhanced permeability of AMPARs to Ca2+, resulting in delayed cell death. However, it is unclear whether the same mechanisms exist in CA3 neurons and whether this underlies the differential sensitivity to ischemia. Here, we investigated the consequences of OGD for AMPAR function in CA3 neurons using electrophysiological recordings in rat hippocampal slices. Following a 15 min OGD protocol, a substantial depression of AMPAR-mediated synaptic transmission was observed at CA3 associational/commissural and mossy fiber synapses but not CA1 Schaffer collateral synapses. The depression of synaptic transmission following OGD was prevented by metabotropic glutamate receptor 1 (mGluR1) or A3 receptor antagonists, indicating a role for both glutamate and adenosine release. Inhibition of PLC, PKC, or chelation of intracellular Ca2+ also prevented the depression of synaptic transmission. Inclusion of peptides to interrupt the interaction between GluA2 and PICK1 or dynamin and amphiphysin prevented the reduction in surface and total AMPAR protein levels after OGD. We also show that a reduction in surface and total AMPAR protein levels after OGD was prevented by mGluR1 or A3 receptor antagonists, indicating that AMPARs are degraded following internalization. Thus, we describe a novel mechanism for the removal of AMPARs in CA3 pyramidal neurons following OGD that has the potential to reduce excitotoxicity and promote neuroprotection.

Introduction

Each region of the hippocampus has a differential resistance to ischemic conditions, with the CA1 being the most susceptible and CA3 and the dentate gyrus most resistant (Kirino et al., 1985; Pulsinelli, 1985; Lipton, 1999). Although mechanisms such as local differences in glutamate receptor expression and sensitivity (Newell et al., 1990; Cronberg et al., 2005; Butler et al., 2010; Sun et al., 2010), synaptic structure (Martone et al., 2000), mitochondrial function (Kass and Lipton, 1986), or Ca2+ influx (Stanika et al., 2010) have been suggested, it remains unclear how, for example, the CA3 region is more resistant to ischemic damage than the neighboring CA1 region.

Ischemia causes a lack of oxygen and glucose, resulting in general metabolic failure that, in turn, leads to a loss of function of Na+/K+ ATP transporters (among many other factors) that maintain the neuronal membrane potential. Failure to maintain the Na+/K+ gradient in the brain prevents glutamate reuptake and causes increased release of glutamate from the cell, resulting in a massive accumulation of extracellular glutamate (Rossi et al., 2000, 2007). The subsequent activation of glutamate receptors coupled with generalized depolarization produces increases in intracellular Ca2+ (Silver and Erecinska, 1990; Tsubokawa et al., 1992; Lipton, 1999), a well documented trigger for cell death (Choi, 1995; Hardingham, 2009). Inhibition of NMDA receptor (NMDAR) function during ischemia can reduce subsequent cell death (Simon et al., 1984; Aarts et al., 2002), and, importantly, activation of Ca2+-permeable AMPA receptors (AMPARs) after ischemia causes delayed cell death (Liu et al., 2004; Noh et al., 2005). Ca2+-permeable AMPARs are not normally present at high levels in CA1 pyramidal neurons, but following ischemia the expression of the GluA2 subunit (that confers Ca2+ impermeability) is reduced leaving predominantly Ca2+-permeable AMPARs (Pellegrini-Giampietro et al., 1992; Tsubokawa et al., 1994; Gorter et al., 1997; Opitz et al., 2000; Noh et al., 2005).

Oxygen/glucose deprivation (OGD) is an established model of ischemia in vitro that can be used to explore potential differences in the response of hippocampal subregions to this insult (Frantseva et al., 1999; Yin et al., 2002; Pugliese et al., 2006; Cimarosti and Henley, 2008; Dixon et al., 2009; Sun et al., 2010). In...
a recent study, we described postsynaptic AMPAR trafficking in CA1 pyramidal neurons following OGD in a process mediated by the PDZ binding protein PICK1 (Dix et al., 2009). It is not known, however, whether this is a general mechanism common to other cell types within the hippocampus.

Here, we demonstrate that a 15 min OGD protocol produces a substantial and persistent depression of AMPAR-mediated responses that is specific to CA3 pyramidal neurons of the hippocampus. This depression of synaptic transmission is a postsynaptic event that is independent of NMDAR and AMPAR activity and does not occur in CA1 pyramidal neurons. We find the depression of transmission is mediated by activation of metabotropic glutamate receptor 1 (mGluR1) and adenosine A3 receptors and leads to AMPAR endocytosis and subsequent degradation.

Materials and Methods

Ethical approval. Animal care and experimental procedures were conducted in accordance with British animal protection legislation and experimental protocols approved by the British National Committee for Ethics in Animal Research.

Slice preparation. Transverse hippocampal slices were prepared from male juvenile (P12–P16) Wistar rats, unless otherwise indicated. Brains were immediately removed following cervical dislocation and immersed in ice-cold aCSF containing the following (in mM): 119 NaCl, 10 glucose, 26 NaHCO3, 2.5 KCl, 1 NaH2PO4, 1 CaCl2, and 5 MgSO4. Individual hippocampi were mounted on agar and 400-μm-thick slices were cut using a DSK 1000 microslicer (DSK). Following dissection, slices were transferred to aCSF containing the following (in mM): 119 NaCl, 10 glucose, 26 NaHCO3, 2.5 KCl, 1 NaH2PO4, 1.3 MgSO4, and 2.5 CaCl2, maintained at 35°C for 30 min, and then stored at room temperature. After dissection, slices were left for a minimum of 1 h before recordings were made. All solutions were saturated with 95% O2 and 5% CO2.

Electrophysiology. Slices were placed in a submerged recording chamber perfused with aCSF (as above) at 35°C with the addition of 50 μM picrotoxin and 5 μM trans-2-carboxy-5,7-dichloro-4-phenoxyacetylaminobenzyl-1,2,3,4-tetrahydroquinoline (L-689,560) unless otherwise stated. A high flow rate of 8–10 ml/min was used to ensure rapid solution exchange times. CA3 pyramidal cells were visualized using infrared–differential interference contrast optics on an Olympus BX-51WI microscope. Patch electrodes with a resistance of 4–5 MΩ were pulled from borosilicate filamented glass capillaries (Harvard Apparatus) using a vertical puller (PC-10; Narishige). Pipettes were filled with intracellular solution containing the following (in mM): 117 CsMeSO4, 8 NaCl, 10 HEPEs, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP, 0.2 EGTA, 0.1 bestatin, and 0.1 leupeptin, set to pH 7.4, 280–285 mOsm.

Records from CA3 pyramidal neurons were made with a Axopatch 200B amplifier (Molecular Devices), filtered at 5 kHz, and digitized at 10 kHz using a data acquisition board and Signal acquisition software (CED). Cells were voltage clamped at −70 mV (without junction potential correction). Series resistance was monitored throughout the experiments, and cells that showed >20% change were discarded from subsequent analysis. Recordings were also rejected from analysis if the series resistance was greater than 30 MΩ. Extracellular field potential recordings were made from hippocampal slices bathed in aCSF using a patch pipette filled with aCSF. The peak amplitude of evoked synaptic responses was measured to calculate response amplitude. Synaptic responses were evoked with 100 μs square voltage steps applied at 0.05 Hz through a biparallel stimulating electrode (FHC) located in stratum radiatum (for associational/commissural or Schaffer collateral stimulation) or the granule cell layer (for mossy fiber stimulation). The recording electrode was placed in stratum radiatum of CA1 (for Schaffer collateral recording) or CA3 (for associational/commissural recording) or in stratum lucidum in CA3 (for mossy fiber recording). Purity of mossy fiber responses was checked by application of the group II mGluR agonist DCV-IV [(5S,2′R,3′R)-2-(2′3′-dicarboxyethylpropionyl)glycine] (2 μM) (Kamiya et al., 1996) at the end of each experiment and if there was <80% depression the experiment was discarded from subsequent analysis (0 of 4 experiments).

Focal application of aCSF containing 10 mM glutamate was performed by pressure ejection (150 ms, 30–90 kPa) under the control of a spritzer (made in house) through a glass electrode with a resistance of 3–4 MΩ placed in stratum radiatum close to the cell body layer.

OGD was induced by perfusing slices with aCSF saturated with 95% N2/5% CO2, and containing 10 mM sucrose instead of 10 mM glucose. L-689,560, kynurenic acid, N-(2-methoxyphenyl)-N′-[2-(3-pyridinyl)-4-quinoxalinyl]-urea (VU5574), 6-amino-N-cyclohexyl-N(3-dimethyl-thiazole[3,2-a]benzimidazole-2-carboxamide hydrochloride (YM298198), (2S)-2-amino-2-[(1S,2S)-2-carboxycyclopent-1-yl]-3-(saxthyl-9)-propanoic acid (LY341495), MPEP, EVK1, SVK1, EVK2, BAFTA, O-(octahydro-4, 7-methano-1H-inden-5-yl)carbonopotasium dihydrate (D609), P4, (R)-3,5-dihydroxyphenylglycine (DHPG), and 1-[2-chloro-6-[[3-iodophenyl]methyl][amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-thio-ribofuranuronamide (2-C1-IB-MECA) were purchased from Tocris. 3-Ethyl-5-benzyl-2-methyl-4-phenylethyl-6-phenyl-1,4-(2-) dihydropyrimidine-3,5-dicarbonylate (MRS1191), picrotoxin, and PKC19-36 were purchased from Sigma-Aldrich.

Slice biotinylation. Acute hippocampal slices (400 μm) were washed once with ice-cold aCSF (5 min) and then incubated with Sulfo-NHS-SS-Biotin (Pierce; 0.5 mg/ml in aCSF) for 30 min on ice. Excess biotin was removed by two brief washes with 50 mM NaCl (in aCSF) and two aCSF washes. The CA1 and CA3 regions were separated and then lysed in 300 μl of ice-cold lysis buffer (150 mM NaCl, 20 mM HEPES, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, pH 7.4) containing protease inhibitors (protease inhibitor mixture with EDTA; Roche). Samples were then sonicated and placed on a head-over-head shaker for 2 h. Samples were then centrifuged at 13,000 rpm for 10 min and the pellets were discarded. The protein concentration of the resulting supernatant was determined using a BCA kit (Pierce).

Streptavidin pull down. Lysed biotinylated samples were added to the streptavidin beads and mixed on a head-over-head shaker for 2 h. Beads were subsequently washed three times with lysis buffer and biotinylated proteins were eluted from the beads using 2× SDS-PAGE loading buffer (containing β-mercaptoethanol) and heated to 90°C for 5 min.

Western blots and densitometry. For Western blot analysis, the CA1 and CA3 regions were isolated with a scalpel from eight hippocampal slices per animal, immersed in lysis buffer containing the following: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, and mammalian protease inhibitor mixture, and homogenized on ice, and the protein concentration was determined. Samples were heated for 5 min at 95°C with 5% β-mercaptoethanol and subjected to SDS-PAGE. The primary antibodies used in Figure 8a were as follows: rabbit polyclonal anti-GluA1 antibody (1:2000; Millipore), mouse polyclonal anti-GluA2/3 antibody (1:1500; Millipore Bioscience Research Agents), and mouse monoclonal anti-β-actin (1:50,000; Sigma-Aldrich). Because the lack of Student’s two-tailed paired t test. Reported tests are within data set unless otherwise stated. Across data sets, statistical comparison was made between 10 min baseline recording and the recording made 20–30 min following the end of OGD or drug application, using a Student’s two-tailed paired t test. For experiments on the effect of intracellular infusion, within-data set comparison was made between the first minute recording and that made 15–20 min after membrane rupture using the lack of Student’s two-tailed paired t test. For experiments comparing the OGD groups treated with each specific drug were compared with non-OGD-exposed groups treated with the respective drugs. All the blots were also probed with anti-β-actin or anti-β-tubulin antibodies as an internal control. The band intensity was quantified by densitometry using NIH Image, and the GluA1/2 values were normalized to β-actin/β-tubulin.

Data analysis. Within an electrophysiological data set, statistical comparison was made between 10 min baseline recording and the recording made 20–30 min following the end of OGD or drug application, using a Student’s two-tailed paired t test. For experiments on the effect of intracellular infusion, within-data set comparison was made between the first minute recording and that made 15–20 min after membrane rupture using the lack of Student’s two-tailed paired t test. Reported tests are within data set unless otherwise stated. Across data sets, statistical comparison was made comparing the normalized data 20–30 min.
OGD induces a depression of AMPA receptor-mediated responses in CA3 neurons

OGD or in vivo ischemia causes a decrease in GluA2 containing and an increase in GluA1 homomeric AMPARs at Schaffer collateral synapses in the CA1 region of the hippocampus (Pellegrini-Giampietro et al., 1992; Tsubokawa et al., 1994; Gorter et al., 1997; Opitz et al., 2000; Noh et al., 2005; Dixon et al., 2009). We therefore tested whether this process also occurs at other hippocampal synapses.

We initially used extracellular field potential recording to assess the effect of 15 min OGD on synaptic transmission recorded (in the presence of NMDAR antagonist L-689,560) at three separate synapses in hippocampal slices: the Schaffer collateral (SC) synapse in CA1, the associational/commissural (AC) synapse in CA3, and the mossy fiber (MF) synapse in CA3. We observed two distinct effects of OGD between synapses of the CA3 and CA1 regions. After obtaining a stable 10 min baseline, a 15 min period of OGD was applied, followed by a 30 min recovery period in which the slice was bathed in oxygen and glucose containing aCSF. OGD resulted in a transient depression of field EPSPs (fEPSPs) recorded at MF, AC, and SC synapses. However, OGD caused a persistent substantial depression of synaptic transmission at CA3 MF and AC synapses but not at CA1 SC synapses (Fig. 1a) (MF, 2 ± 2%, n = 4, p < 0.001; AC, 12 ± 4%, n = 5, p < 0.05; SC, 78 ± 12%, n = 4, p > 0.05). As juvenile rats are less susceptible to ischemic damage than adult rats (Popa-Wagner et al., 2007), we performed the same experiment on hippocampal slices obtained from P35–P45 rats. The same substantial depression of synaptic transmission at AC synapses in the CA3 region was observed following OGD (Fig. 1b) (6 ± 8%; n = 5; p < 0.01). Extracellularly recorded fiber volleys revealed a transient depression in fiber volley amplitude during OGD that recovered to baseline within 30 min at both AC and MF synapses (Fig. 1c) (MF, 98 ± 10%, n = 9, p > 0.05; AC, 103 ± 18%, n = 7, p > 0.05). These data indicate the transient depression during OGD, but not the sustained depression of synaptic transmission following OGD, is due to an inhibition of presynaptic action potentials. We also applied 15 min of OGD to hippocampal slices in the absence of any AMPAR, NMDAR, or GABA_A receptor antagonists. The same depression in fEPSP amplitude was observed (Fig. 1d) (2 ± 7%; n = 7; p < 0.001), indicating that the depression of synaptic transmission in CA3 occurred in the presence or absence of NMDAR antagonists.

We observed the same depression of synaptic transmission when making whole-cell voltage-clamp recordings from CA3 pyramidal neurons and stimulating AC fibers in stratum radiatum. In the presence of NMDAR and GABA_A receptor antagonists following the end of OGD or drug application using a Student’s unpaired two-tailed t test.

Within Western blot data sets, statistical comparisons were made between normalized control and OGD conditions using one-sample two-tailed t tests. Subsequent comparisons between OGD, OGD plus MRS1191, and OGD plus YM298198 were made using one-way ANOVA with Bonferroni’s post hoc correction. All data are expressed as mean ± SEM. Values of p < 0.05 were considered statistically significant.

Results

OGD induces a depression of AMPA receptor-mediated responses in CA3 neurons

Figure 1.
(L-689,560 and picrotoxin, respectively), AMPAR-mediated EPSCs were substantially depressed following 15 min OGD (Fig. 1e) (15 min, 15 ± 6%; n = 7; p < 0.001). Shorter durations of OGD produced smaller reductions in AMPAR EPSC amplitude (Fig. 1e) (10 min, 43 ± 12%; n = 11, p < 0.05; 5 min, 95 ± 6%; n = 7, p > 0.05). During in vivo ischemia or OGD, a delayed, but rapid-onset, depolarization has been observed, which recovers with reperfusion or reapplication of glucose and oxygen (Hansen, 1985; Rossi et al., 2000; Joshi and Andrew, 2001). Toward the end of OGD exposure, we consistently observed a rapid increase in holding current that corresponds to this depolarization (average for 15 min OGD, 758 ± 138 pA; n = 7).

The depression of synaptic transmission could be due to impaired presynaptic glutamate release or a reduction in postsynaptic responsiveness. To distinguish between these possibilities, we bypassed the presynaptic release of neurotransmitter using application of exogenous glutamate (10 mM) by pressure ejection from a glass pipette placed in stratum radiatum, while making whole-cell voltage-clamp recordings from CA3 pyramidal neurons. Glutamate-evoked currents in the presence of the NMDAR antagonist L-689,560 (5 μM) and the GABA<sub>A</sub> receptor antagonist picrotoxin (50 μM) displayed a persistent reduction following OGD (Fig. 1f) (2 ± 1%; n = 7; p < 0.001). The same result was observed when the experiment was performed in the absence of picrotoxin (3 ± 1%; n = 8; p < 0.001) (data not shown).

We also tested whether the depression of synaptic transmission on CA3 pyramidal neurons was specific for AMPAR-mediated transmission. NMDAR EPSCs were evoked by stimulation of the AC fibers, while recording from CA3 pyramidal neurons voltage clamped at +40 mV in the presence of NBQX (20 μM) and picrotoxin (50 μM) to block AMPAR and GABA<sub>A</sub>R, respectively. After a transient decrease in the amplitude of NMDAR EPSCs during OGD, there was a substantial recovery following OGD, demonstrating that the long-term depression of synaptic transmission is specific for AMPAR-mediated transmission (Fig. 1g) (74 ± 7%; n = 11; p > 0.05). These data suggest the depression of synaptic transmission in CA3 pyramidal neurons following OGD is a postsynaptic event resulting from a removal or inactivation of AMPARs at both synaptic and extrasynaptic sites.

**OGD-induced depression of synaptic transmission requires mGluR1 but not AMPAR or NMDAR activation**

Metabolic failure results in prolonged excess glutamate release during OGD, which leads to glutamate receptor activation (Rossi et al., 2000). We therefore tested a range of antagonists to determine which, if any, glutamate receptors mediated the depression of synaptic transmission at the AC synapse in CA3 after OGD. Our recordings in Figure 1, a, b, c, and f, were all performed in the presence of an NMDAR antagonist, indicating the depression of synaptic transmission after OGD is not NMDAR dependent. To test whether AMPA or kainate receptor activation is required for the depression of synaptic transmission following OGD, we used the low-affinity AMPA/kainate receptor antagonist kynurenic acid. A 15 min application of 2 mM kynurenic acid substantially reduced AMPAR EPSCs at AC synapses recorded in the presence of NMDAR and GABA<sub>A</sub> receptor antagonists L-689,560 and picrotoxin (Fig. 2a) (20 ± 4%; n = 5; p < 0.05), which then fully recovered within 20 min of wash out (93 ± 12%; n = 5; p > 0.05 compared with baseline). The 2 mM kynurenic acid was then applied for the 15 min period of OGD to inhibit AMPAR activity. Inhibition of AMPARs during OGD failed to prevent the depression of synaptic transmission following OGD (Fig. 2b) (12 ± 3%; n = 8; p < 0.001), suggesting that direct agonist activation of AMPARs during OGD is not the primary mechanism underlying the decrease in functional AMPARs.

We next investigated whether mGluRs could mediate the depression of synaptic transmission. There are eight known mGluR subtypes that are classified into three groups. To test a role for mGluRs in the depression of AMPAR EPSCs following OGD, the mGluR antagonist LY341495 (100 μM) was applied during OGD. At this concentration, all mGluR subtypes are blocked by this antagonist. LY341495 prevented the long-term depression of synaptic transmission following OGD, demonstrating a role for mGluRs (Fig. 2c) (85 ± 18%; n = 6; p > 0.05). Group I mGluRs
consist of mGluR1 and mGluR5, which are predominantly found postsynaptically within the hippocampus where they mediate a form of LTD and are thus likely candidates to mediate the depression of synaptic transmission following OGD (Moult et al., 2006; Lüscher and Huber, 2010). Application of the mGluR5 antagonist MPEP (30 μM) failed to prevent the depression of AMPAR EPSCs following OGD (Fig. 2d) (5 ± 2%; n = 6; p < 0.001), suggesting that mGluR5 activation is not involved. However, application of the mGluR1 antagonist YM298198 (50 nM) during OGD did prevent the depression of AMPAR EPSCs following OGD (Fig. 2e) (91 ± 4%; n = 7; p > 0.05). Importantly, application of 50 nM YM298198 alone did not result in any change in EPSC amplitude or paired-pulse ratio (PPR) (Fig. 2f) (92 ± 7%; PPR, 108 ± 5%; n = 7; p > 0.05 for both), suggesting that inhibition of mGluR1 does not modulate AMPAR-mediated responses or change postsynaptic probability of release under control conditions.

OGD-induced depression of synaptic transmission requires adenosine A$_1$ receptor activation

Under stress conditions, such as those evoked during ischemia and OGD, high levels of adenosine are released within the hippocampus (Latini et al., 1999; Latini and Pedata, 2001). Of the four adenosine receptor subtypes, A$_1$, A$_2a$, A$_2b$, and A$_3$, A$_1$ and A$_3$ receptors are predominantly located presynaptically and affect release probability at excitatory synapses and A$_2b$ is absent from the hippocampus (Cunha et al., 1994; Swanson et al., 1995; Dixon et al., 1996; Dunwiddie and Masino, 2001; Rebola et al., 2005). A$_1$ receptors are present in the hippocampus, but their presynaptic or postsynaptic location remains unclear. Similar to mGluR1, A$_3$ receptors are G-protein-coupled receptors, typically linked to G$_q$ and G$_i/o$ affecting phospholipase C (PLC) and adenyl cyclase activity. Interestingly, A$_3$ receptor activation appears to be neuroprotective during ischemia, although a precise role is unclear (von Lubitz et al., 1999; Pugiøese et al., 2006).

Consistent with this, application of the A$_1$ antagonist VUF5574 (100 nM) during OGD prevented the depression of AMPAR EPSCs at AC synapses following OGD (Fig. 3a) (100 ± 21%; n = 6; p > 0.05). To confirm the requirement for A$_1$ receptor activation, another A$_1$ antagonist MRS1191 (1 μM) was applied during OGD that also prevented the depression of AMPAR EPSCs (Fig. 3b) (83 ± 7%; n = 7; p > 0.05). Application of either antagonist alone without OGD did not change the AMPAR EPSC amplitude or PPR (Fig. 3c) (VUF, 93 ± 10%; PPR, 114 ± 8%; n = 6; p > 0.05 for both; MRS, 87 ± 9%; PPR, 99 ± 10%; n = 7; p > 0.05 for both). These experiments indicate that A$_1$ receptor activation during OGD is required for the depression of AMPAR EPSCs following OGD.

Activation of mGluR1 and A$_3$ receptors is not sufficient for complete depression of synaptic transmission following OGD

The results so far demonstrate that mGluR1 and A$_3$ receptor activation during OGD is necessary for the long-term depression of AMPAR EPSCs. Therefore, we next determined whether activation of mGluR1 and A$_3$ receptors alone is also sufficient to elicit the depression of AMPAR EPSCs by application of the group I mGluR agonist DHPG and the A$_3$ agonist 2-Cl-IB-MECA. A 15 min application of DHPG (50 μM) resulted in a depression of AMPAR EPSCs without any effect on PPR (Fig. 4a) (56 ± 11%; n = 6; p < 0.05; PPR, 124 ± 15%; p > 0.05). Similarly, application of 2-Cl-IB-MECA (1 μM) also caused a depression of AMPAR EPSCs without any effect on PPR (Fig. 4b) (61 ± 8%; n = 7; p < 0.05; PPR, 122 ± 8%; p > 0.05). However, submaximal concentrations of DHPG (50 nM) and 2-Cl-IB-MECA (500 nM) produced no depression of AMPAR EPSCs (Fig. 4c,d) (107 ± 11%; n = 6, p > 0.05; 111 ± 22%, n = 6, p > 0.05), but when both agonists were applied conjointly at the same submaximal concentrations a depression of AMPAR EPSCs was observed (Fig. 4e) (42 ± 3%; n = 6; p < 0.01). This indicates that mGluR1 and A$_3$ receptors can act in a complementary fashion to depress synaptic transmission at AC synapses in CA3. We next tested whether conjoint application of supramaximal concentrations of DHPG and 2-Cl-IB-MECA could reproduce the substantial depression of AMPAR EPSCs seen after OGD. DHPG (50 μM) and 2-Cl-IB-MECA (1 μM) reduced AMPAR EPSC amplitude only to the same level as submaximal concentrations (Fig. 4f) (43 ± 10%; n = 9; p < 0.01; PPR = 117 ± 10%; p > 0.05), indicating that simultaneous activation of mGluR1 and A$_3$ receptors is not sufficient for the substantial depression of synaptic transmission following OGD.

OGD causes release of glutamate and adenosine and also depolarization of neurons. Therefore, we tested whether the combination of all three events could recapitulate the effects of OGD on synaptic transmission. The DHPG (50 μM)- and 2-Cl-IB-MECA (1 μM)-induced depression was enhanced by depolarizing the postsynaptic cell to 0 mV during application of the mGluR1 and A$_3$ receptor agonists (Fig. 4g) (26 ± 6%; n = 6; p < 0.01). The 15 min depolarization was delayed 5 min compared with agonist application to ensure agonist presence during depolarization. The level of depression induced by DHPG, 2-Cl-IB-MECA, and postsynaptic depolarization was indistinguishable to that in-
Application of the group I mGluR agonist DHPG (50 μM) produced no change in AMPAR EPSCs. Interestingly, although a supramaximal dose of the A₁ agonist 2-Cl-IB-MECA (1 μM) produced a depression of synaptic transmission at AC synapses in CA3, there was no effect on Schaffer collateral synapses in CA1 (Fig. 4h) (90 ± 4%; n = 7; p > 0.05). This suggests that A₁ receptors may be absent from CA1 cells and thus provides a possible explanation for the difference in response to OGD between hippocampal subregions.

OGD-induced depression of synaptic transmission requires intracellular calcium and is mediated by PKC and PLC activity

In vivo ischemia or OGD evoke rises in intracellular Ca²⁺ concentration (Silver and Erecinska, 1990; Lipton, 1999). To test whether this is important for the depression of AMPAR EPSCs following OGD, two experimental approaches were used. The first used removal of Ca²⁺ from aCSF for the period during and 15 min after OGD. This prevented the depression of AMPAR EPSCs following OGD (Fig. 5a) (70 ± 6%; n = 7; p > 0.05; p < 0.05 compared with OGD in the presence of Ca²⁺). The second experiment used the Ca²⁺ chelator BAPTA included in the patch pipette. Inclusion of 30 mM but not 10 mM BAPTA also prevented the depression of AMPAR EPSCs at AC synapses following OGD (Fig. 5b) (50 mM, 83 ± 10%; n = 9, p > 0.05; 10 mM, 5 ± 16%, n = 7, p < 0.001). The high concentration of BAPTA required to buffer a rise in intracellular Ca²⁺ suggests, as previously reported, that ischemia or OGD causes a substantial increase in intracellular Ca²⁺ that is required for the depression of synaptic transmission following OGD.

Both mGluR1 and A₁ receptors are coupled to G-proteins that activate PLC, which subsequently results in two major pathways of intracellular signaling. PLC, via inositol triphosphate (IP₃) activation, leads to the release of Ca²⁺ from intracellular stores (Abbracchio et al., 1995; Palmer et al., 1995) and, via the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂), produces diacylglycerol (DAG), which in turn activates protein kinase C (PKC) (Abbracchio et al., 1995). To determine whether these downstream signaling pathways mediate the depression of synaptic transmission following OGD, both PKC and PLC inhibitors were used. Inclusion of the PLC inhibitor D609 (30 μM) in the patch pipette prevented the depression of AMPAR EPSCs at AC synapses following OGD (Fig. 5c) (80 ± 10%; n = 7; p > 0.05). Similarly, inclusion of the PKC inhibitory peptide PKC19-36 (4.8 μM) in the patch pipette also prevented the depression of AMPAR EPSCs (Fig. 5e) (84 ± 10%; n = 7; p > 0.05). Recordings made immediately following membrane rupture demonstrate that PKC inhibition by inclusion of PKC19-36 in the patch pipette had no effect on AMPAR EPSC amplitude (Fig. 5f) (108 ± 14%; n = 6; p > 0.05). However, inclusion of D609 in the patch pipette showed that PLC inhibition increased AMPAR EPSC amplitude (Fig. 5d) (182 ± 41%; n = 7; p < 0.05). In this case, a stable baseline was achieved before application of OGD. These data indicate that both PLC and PKC play a role in mediating the depression of synaptic transmission following OGD.

Depression of synaptic transmission following OGD requires PICK1-mediated and dynamin-dependent AMPAR internalization

The protein PICK1 is critical for the removal of AMPARs from the synaptic membrane during LTD (Hanley, 2008; Terashima et al., 2009). The high concentration of BAPTA required to buffer a rise in intracellular Ca²⁺ suggests, as previously reported, that ischemia or OGD causes a substantial increase in intracellular Ca²⁺ that is required for the depression of synaptic transmission following OGD.

Both mGluR1 and A₁ receptors are coupled to G-proteins that activate PLC, which subsequently results in two major pathways of intracellular signaling. PLC, via inositol triphosphate (IP₃) activation, leads to the release of Ca²⁺ from intracellular stores (Abbracchio et al., 1995; Palmer et al., 1995) and, via the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂), produces diacylglycerol (DAG), which in turn activates protein kinase C (PKC) (Abbracchio et al., 1995). To determine whether these downstream signaling pathways mediate the depression of synaptic transmission following OGD, both PKC and PLC inhibitors were used. Inclusion of the PLC inhibitor D609 (30 μM) in the patch pipette prevented the depression of AMPAR EPSCs at AC synapses following OGD (Fig. 5c) (80 ± 10%; n = 7; p > 0.05). Similarly, inclusion of the PKC inhibitory peptide PKC19-36 (4.8 μM) in the patch pipette also prevented the depression of AMPAR EPSCs (Fig. 5e) (84 ± 10%; n = 7; p > 0.05). Recordings made immediately following membrane rupture demonstrate that PKC inhibition by inclusion of PKC19-36 in the patch pipette had no effect on AMPAR EPSC amplitude (Fig. 5f) (108 ± 14%; n = 6; p > 0.05). However, inclusion of D609 in the patch pipette showed that PLC inhibition increased AMPAR EPSC amplitude (Fig. 5d) (182 ± 41%; n = 7; p < 0.05). In this case, a stable baseline was achieved before application of OGD. These data indicate that both PLC and PKC play a role in mediating the depression of synaptic transmission following OGD.

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OGD-induced depression of AMPAR EPSCs requires intracellular Ca$^{2+}$, PLC, and PKC activation. a, Removal of Ca$^{2+}$ from aCSF during OGD and for a further 15 min prevented the depression of AMPAR EPSCs. b, Inclusion of 50 mM but not 10 mM BAPTA to the patch pipette prevented the depression of AMPAR EPSCs. c, Inclusion of the PLC inhibitor D609 (30 μM) in the patch pipette prevented the depression of AMPAR EPSCs. d, Inclusion of 30 μM D609 in the patch pipette increased AMPAR EPSCs without changing PPR. e, Inclusion of the PKC inhibitory peptide PKC19–36 (4.8 μM) in the patch pipette prevented the depression of AMPAR EPSCs. f, Inclusion of 4.8 μM PKC19–36 in the patch pipette had no effect on AMPAR EPSCs or PPR. The gray boxes indicate period of OGD. Example traces illustrate responses before (black, green, or blue) and 20–30 min following OGD (red). Calibration: 50 pA, 20 ms. Error bars indicate SEM.

Figure 5.

OGD leads to a decrease of AMPAR protein levels specific to the CA3 region of the hippocampus

To confirm that AMPARs are internalized from the plasma membrane following OGD, we performed surface biotinylations for GluA1 and GluA2. Hippocampal slices were either exposed to 15 min OGD or kept as control in aCSF containing oxygen and glucose. Thirty minutes following OGD, slices were labeled with biotin before the CA3 and CA1 regions were separated and subsequently processed for streptavidin chromatography and Western blotting. There was no difference in amounts of surface GluA1 or GluA2 subunits between OGD and controls within the CA1 region (Fig. 7a,b) (GluA1 OGD, 96 ± 10%, n = 6, p > 0.05; GluA2 OGD, 123 ± 26%, n = 6, p > 0.05).
However, there was a reduction in surface GluA1 and GluA2 subunits within the CA3 region in slices that had received OGD (Fig. 7c, d) (GluA1 OGD, 61 ± 5%, n = 6, p < 0.05; GluA2 OGD, 58 ± 6%, n = 6, p < 0.05).

mGluR1 and A3 receptors are both required for the removal of synaptic AMPARs following OGD (Figs. 2–4), so we tested whether their activation is also required for the reduction in surface levels of AMPAR subunits observed in the biotinylation assay. Hippocampal slices were subjected to 15 min OGD or control conditions in the presence of the mGluR1 antagonist YM298198 (50 nM) or the A3 receptor antagonist MRS1191 (1 μM), and surface biotinylations were performed. The mGluR1 antagonist prevented the reduction in surface GluA1 and GluA2 (Fig. 7c, d) (GluA1 OGD, 114 ± 10%, n = 6, p > 0.05; GluA2 OGD, 94 ± 9%, n = 6, p > 0.05), and the A3 receptor antagonist partially prevented the reduction in surface GluA1 and GluA2 (Fig. 7c, d) (GluA1 OGD, 80 ± 3%, n = 6, p < 0.05; GluA2 OGD, 79 ± 6%, p < 0.05).

Figure 6. OGD induces PICK1 and dynamin-dependent internalization of AMPARs. a, Inclusion of either pep2-SVKI (100 μM) or pep2-EVKI (100 μM) but not pep2-SVKE (100 μM) in the patch pipette prevented the depression of AMPAR EPSCs following OGD. Inclusion of 100 μM SVKI (c) or EVKI (d) but not SVKE peptide (b) in the patch pipette increased AMPAR EPSCs. e, Inclusion of the dynamin inhibitory peptide P4 (50 μM) in the patch pipette prevented the depression of AMPAR EPSCs following OGD. f, Inclusion of the dynamin inhibitory peptide P4 (50 μM) in the patch pipette prevented the depression of glutamate-evoked (10 μM) responses following OGD. The gray boxes indicate 15 min period of OGD. Example traces illustrate responses before (black, green, or blue) and 20–30 min following OGD or 10–15 min after beginning the experiment (red). Calibration: 50 pA, 10 or 200 ms (g). Error bars indicate SEM.

Figure 7. OGD leads to a decrease of surface AMPARs in the CA3 region. a, b, Surface GluA1 and GluA2 levels were decreased in CA3 but not CA1 following OGD. Control and OGD exposed slices were subject to surface biotinylation. The relative surface (S) and total (T) AMPARs was assessed by Western blot using specific antibodies against GluA1 and GluA2. Representative blots of 50% total and 100% surface GluA1 and GluA2 subunits and tubulin after OGD in CA1 region and 25% total and 100% surface in CA3 region. Graphs show the ratio of surface biotinylated to total AMPARs expressed as a percentage of control. c, d, Inhibition of mGluR1 or A3 receptors prevented or attenuated the decrease in surface GluA1 and GluA2 in CA3 following OGD. Incubation of the slices in YM298198 and MRS1191 during OGD produced less internalization of AMPARs than control OGD-exposed slices. *p < 0.05 and ns (not significant, p = 0.07 for GluA1 and p = 0.05 for GluA2) indicate statistical tests against control OGD; #p < 0.05 and ##p < 0.01, significant difference to control in the absence of OGD. Error bars indicate SEM.
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Discussion

In this study, we demonstrate that OGD induces an almost complete depression of AMPAR-mediated synaptic transmission onto CA3 pyramidal neurons of the hippocampus. This effect in slices taken from young animals is caused by the coactivation of mGluR1 and A3 receptors together with a rise in intracellular calcium concentration leading to the removal of surface AMPARs.

The observed depression of AMPAR EPSCs following OGD could potentially result from a variety of sources, including depression of presynaptic action potentials, depression of presynaptic neurotransmitter release, or removal of postsynaptic receptors. We conclude the depression of synaptic transmission results from a removal of postsynaptic AMPARs for the following reasons: (1) presynaptic fiber volley does not decrease following OGD (Fig. 1c); (2) responses elicited by exogenous glutamate application are depressed by OGD (Fig. 1f); (3) NMDAR EPSCs recover following OGD (Fig. 1g); (4) specific manipulation of the postsynaptic environment by the introduction of BAPTA, PKC19-36, or D609 could reverse the depression of synaptic transmission (Fig. 5); (5) interfering with specific protein interactions known to regulate AMPAR internalization (PICK1 PDZ domain interactions and dynamin–amphiphysin interactions) prevents the depression of AMPAR-mediated synaptic transmission (Fig. 6). Furthermore, the involvement of PICK1 and dynamin in AMPAR EPSC depression suggests this event is an internalization of AMPARs mediated by endocytosis. This conclusion is supported by surface biotinylation data showing a decrease in the surface expression of GluA1 and GluA2 following OGD (Fig. 7).

Furthermore, the data suggest that internalization is followed by degradation of AMPAR subunits, which is selective to the CA3 hippocampal region (Fig. 8). Our electrophysiological data show a complete removal of AMPARs from CA3 neurons following OGD (Fig. 1), whereas the surface biotinylation data show only a ~40% decrease (Fig. 7). This is perhaps not surprising since surface biotinylation will sample AMPARs on all cell types in CA3 and CA1 that we previously observed using electrophysiological techniques (Dixon et al., 2009). We observe degradation of both GluA1 and GluA2 in the CA3 region 1 h following OGD. It is not currently clear whether all AMPARs are eventually degraded in CA3 pyramidal neurons following OGD or only the ~30% that we show 1 h after OGD (Fig. 8). Our data suggest that OGD in CA3 results in a general endocytosis followed by degradation of AMPARs rather than switching between specific subunits as previously observed in the CA1 region (Noh et al., 2005; Dixon et al., 2009). Using biotinylation assays, we do not observe the OGD-dependent internalization of GluA2 in CA1 that we previously observed using electrophysiological techniques (Dixon et al., 2009). As discussed above, this could be explained by the contribution of AMPARs expressed on interneurons and glial cells to the biotinylation assay, masking the relatively subtle effect that might be expected following 15 min OGD in CA1 (Dixon et al., 2009).
Activity-dependent plasticity of postsynaptic glutamate receptors in CA3 pyramidal neurons can be mediated by a variety of mechanisms. Long-term potentiation (LTP) of AMPAR EPSCs at AC synapses requires NMDAR activation but specifically not mGluR1 (Kobayashi and Poo, 2004), whereas LTD of AMPAR EPSCs at MF synapses requires Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels instead of NMDAR or mGluR activation (Lei et al., 2003). This form of MF LTD requires the presence of PICK1, implying that AMPARs are removed from the synaptic membrane (Ho et al., 2009). LTD of kainate receptors at MF synapses also requires PICK1 as well as mGluR5 and PKC activation but not an increase in internal Ca\(^{2+}\) in a process that removes synaptic receptors from the plasma membrane (Selak et al., 2009). Finally, LTP of NMDARs at MF synapses requires activation of adenosine A\(_2\)A, NMDA, and mGluR5 receptors coupled to PKC activation (Kwon and Castillo, 2008; Rebola et al., 2008). Thus, evidence to date suggests there are multiple signaling pathways for the insertion or removal of glutamate receptors from the postsynaptic membrane in CA3 pyramidal neurons with common elements to many of them. The mechanisms for the removal of AMPARs following OGD demonstrated in this study illustrate how the receptors are subsequently targeted for degradation rather than being recycled back to the plasma membrane (Lee et al., 2004).

Both mGluR1 and A\(_3\) receptors couple to the G\(_{4}\) subtype of G-protein (Nakamura et al., 1994; Abbraccchio et al., 1995). Our data support a mechanism in which the G\(_{4}\)-coupled signaling pathway leads to activation of PLC, which cleaves PIP\(_2\) into DAG and IP\(_3\). Activation of IP\(_3\) receptors on the endoplasmic reticulum causes release of intracellular Ca\(^{2+}\) stores while DAG activates PKC (Abbraccchio et al., 1995). It has been shown that phosphorylation of GluA2 by PKC coupled with an increase in intracellular Ca\(^{2+}\) concentration promotes trafficking of GluA2 containing AMPARs by PICK1 (Xia et al., 2000; Perez et al., 2001; Hanley and Henley, 2005) and subsequent dynamin-dependent endocytosis. We propose this is the mechanism for the removal of AMPARs from the surface membrane, but our results do not indicate how the receptors are subsequently targeted for degradation rather than being recycled back to the plasma membrane (Lee et al., 2004).

Since mGluR1 and A\(_3\) receptors both couple to the same G\(_{4}\)-initiated signaling pathway, this suggests the same pool of G-proteins are activated by each receptor. However, both mGluR1 and A\(_3\) receptors are required for the removal of AMPARs following OGD so mGluR1 activation should compensate for A\(_3\) receptor stimulation and vice versa. Our data do not support this interpretation, implying that separate signaling pathways are used by each receptor. Alternatively, mGluR1 and A\(_3\) receptors could form a more direct G-protein-independent interaction (Tabata et al., 2007).

Although supramaximal concentrations of agonists of either mGluR1 or A\(_3\) receptors alone can induce LTD in CA3, when we used lower submaximal concentrations we found that activation of both receptors at the same time produced LTD, whereas each on their own did not. This demonstrates a cooperative action between the two receptors, and when this was coupled with postsynaptic depolarization, which will produce calcium influx via voltage-dependent calcium channels, we were able to recapitulate the depression of synaptic transmission induced by OGD. This indicates that these three events are sufficient to cause the removal of AMPARs from the plasma membrane during OGD.

The removal of surface AMPARs from CA3 pyramidal neurons following OGD is antici excitatory and could therefore reduce excitotoxicity in the hippocampus after ischemic insult. This provides mGluR1 and A\(_3\) receptors with a potentially critical role in regulating cell death following ischemia. The A\(_3\) receptor is suited to this role since its affinity for adenosine is low and will only be activated by the high levels of adenosine released during ischemia (Latini et al., 1999; von Lubitz et al., 1999). In addition, A\(_3\) receptor activation has been shown to promote the recovery of synaptic responses following OGD in the CA1 region (Pugliese et al., 2006). However, reports are unclear and contrasting on the neuroprotective effects of A\(_3\) receptor activation during ischemia (von Lubitz et al., 1999). Activation of mGluR1 induces a postsynaptic LTD in CA1 pyramidal neurons mediated by PKC and PICK1 (Moult et al., 2006; Niehusmann et al., 2010), and there is also evidence that mGluR1 activation is neuroprotective (Kohara et al., 2008). Our data support a neuroprotective role for mGluR1 or A\(_3\) receptor activation.

The reasons why OGD-induced depression of synaptic transmission occurs in the CA3 but not CA1 region is unclear. Our data suggest the presence of adenosine A\(_3\) receptors could be a critical factor since A\(_3\) receptor activation depressed synaptic transmission in CA3 but not in CA1 (Fig. 4). However, our data do not distinguish between a lack of A\(_3\) receptors in CA1 or a lack of the coupling between A\(_3\) receptors and AMPAR internalization. Of the other two necessary components to OGD-induced depression of synaptic transmission in CA3, mGluR1-mediated LTD (Moult et al., 2006; Lüscher and Huber, 2010) and OGD-induced depolarization and Ca\(^{2+}\) influx (Rossi et al., 2000, 2007) are both well documented in CA1 pyramidal neurons and are therefore unlikely to explain the observed difference in response to OGD between CA3 and CA1. An alternative possibility is local differences in the coupling of A\(_3\) or mGluR1 receptors to their associated G-protein receptors.

Our data demonstrate clear differences in the response to OGD between CA3 and CA1 pyramidal neurons that support previous findings that CA3 neurons are much better equipped to survive ischemic trauma than CA1 neurons. Understanding the mechanisms that enable CA3 neurons to have a greater resistance to ischemic damage than CA1 neurons could potentially inform future neuroprotective strategies.

References


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