Prolonged positive modulation of AMPA receptors induces calpain-mediated PDZ protein degradation and AMPA receptor down-regulation in cultured hippocampal slices.

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List of abbreviations:
ABP/GRIP2, AMPAr binding protein / glutamate receptor interacting protein 2
AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAr, AMPA receptor
AP-5, 2-amino-5-phosphonopentanoic acid
BDNF, brain-derived neurotrophic factor
CaMKII, calcium-calmodulin-dependent protein kinase II
CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione
cRNA, complementary RNA
CX614, 2H,3H, 6aH-pyrrolidino[2",1"-3',2']1,3-oxazino[6',5'-5,4]benzo[1,4]dioxan 10-one
DMSO, dimethyl sulfoxide
GRIP1, glutamate receptor interacting protein 1
LDH, lactate dehydrogenase
LTD, long term depression
LTP, long term potentiation
mEPSP, miniature excitatory postsynaptic potential
mRNA, messenger RNA
NMDA, N-methyl-D-aspartic acid
NMDAr, NMDA receptor
PDZ, PSD-95 / Dlg /ZO-1
Pick1, protein interacting with C kinase 1
PSD-95, postsynaptic density protein 95
SAP97, synapse-associated protein 97
VSCC, voltage-sensitive calcium channel

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ABSTRACT

Prolonged exposure of cultured hippocampal slices to CX614, a positive AMPA receptor (AMPAr) modulator, decreases receptor response to synaptic stimulation, an effect that could reflect reduced receptor expression. The present study investigates this down-regulation and its underlying mechanisms using cultured rat hippocampal slices. Chronic treatment with CX614 gradually reduced levels of GluR1 and GluR2/3 AMPAr subunits and of their anchoring proteins, SAP97 and GRIP1 through 48 h. Decline in SAP97 and GRIP1 levels was associated with increased abundance of lower molecular weight bands, suggesting degradation of these proteins. CX614 effects were partially reversible following drug removal. GluR1 and GluR2/3 down-regulation and their slow recovery were associated with similar changes in SAP97 and GRIP1 levels. Treatment with CX614 for 48 h significantly reduced AMPAr mRNA levels in hippocampus whereas 8 h exposure did not. Blockade of ionotropic glutamate receptors prevented CX614-induced decrease in AMPAr subunits and mRNA, with regional selectivity, although an AMPAr blocker was more efficacious than an NMDAr blocker. Blockade of calpain activity reduced CX614-induced degradation of SAP97 and GRIP1 and prevented decreases in AMPAr subunit but not mRNA levels. Treatment with CX614 alone or in combination with glutamate receptor blockers or calpain inhibitor III did not modify lactate dehydrogenase release into culture medium, implying the absence of cell toxicity. We conclude that CX614-induced AMPAr protein loss is primarily mediated by AMPAr activation, and involves calpain-dependent proteolysis of SAP97 and GRIP1. CX614-induced suppression of AMPAr gene expression is, however, calpain-independent and all these effects are not associated with cell damage.
INTRODUCTION

AMPA-type glutamate receptors (AMPAr) mediate most of the fast excitatory neurotransmission in the mammalian central nervous system. Several recent studies have implicated the cycling of these receptors into and out of the synaptic compartment in important neurophysiological phenomena such as long-term potentiation (LTP) and long-term depression (LTD) in support of our initial hypothesis (Baudry and Lynch, 1981). AMPAr interactions with distinct scaffolding PDZ proteins regulate the intracellular trafficking and localization of the receptors. For instance, SAP97 and PSD-95 are important for targeting the receptors to synaptic membranes, Pick1 is involved in receptor internalization, and SAP97 and GRIP1 contribute to the stabilization of receptor subunits and to receptor trafficking between different subcellular compartments (Iwakura et al. 2001; Jourdi et al., 2003; Ahmadian et al., 2004; Collingridge et al., 2004 and references therein). Moreover, treatment of neurons with glutamate, NMDA, or brain-derived neurotrophic factor (BDNF) results in translocation of AMPAr to the cell surface via a mechanism involving interaction of the AMPAr subunit GluR2 with the N-ethylmaleimide sensitive factor (NSF) (Broutman and Baudry, 2001; Braithwaite et al., 2002; Narisawa-Saito et al., 2002; Collingridge et al., 2004).

CX614 belongs to the benzamide family of positive AMPA receptor modulators (a.k.a., ampakines). These drugs up–regulate AMPAr-mediated electrophysiological responses to glutamate, increase mEPSC frequency and amplitude, and facilitate LTP (Arai et al., 2000, 2002a, b; Suppiramaniam et al., 2001). CX614 increases the amplitude of AMPAr-mediated currents, prolongs the “open-channel” state of the receptor and increases the frequency and amplitude of mEPSP. However, prolonged exposure to
CX614 results in desensitization and/or run-down of the receptor response (Arai et al., 2000). Such AMPAR down-regulation could possibly reflect enhanced AMPAR internalization and/or removal of the receptors from the synaptic pool.

Calpain is a Ca++-dependent protease present in the synaptic compartment. Its activation is required for the induction of LTP and occurs following incubation of neurons with NMDA, glutamate, and theta burst stimulation (Oliver et al., 1989; Muller et al., 1995; Bednarski et al., 1995; Vanderklish et al., 1996). Conversely, suppression of calpain expression or activity blocks LTP (Oliver et al., 1989; Denny et al., 1990; Bednarski et al., 1995; Vanderklish et al., 1996). Moreover, calpain truncates the C-terminal domains of several AMPA and NMDA receptor subunits, as well as PSD-95 and GRIP1, members of the PDZ family of proteins, (Lu et al., 2000; 2001), and the cytoskeletal protein spectrin (Baudry and Lynch, 1981). In a recent report, we showed that prolonged incubation of cultured hippocampal slices with CX614 resulted in calpain activation and calpain-mediated proteolysis of spectrin (Jourdi et al., 2005). Together, these studies indicate that calpain plays a critical role in the reorganization of glutamatergic postsynaptic compartment following a number of stimuli resulting in increased calcium concentrations in postsynaptic structures. They also suggest that calpain-mediated proteolysis of AMPAR-associated PDZ proteins might be involved in down-regulation of AMPARs. The possibility that prolonged exposure of cultured hippocampal slices to CX614 could lead to the destabilization of AMPAR complexes and the elimination of the receptor subunits was addressed in the present study.
METHODS

Hippocampal slice cultures. Ten to 12 day-old rat pups were obtained from Jackson Laboratories (Bar Harbor, Maine) and used to prepare hippocampal slice cultures as previously described (Jourdi et al., 2005). Briefly, animals were decapitated and the brains were dissected, trimmed, and 400 µm thick hippocampal slices were cut using a McIlwain tissue slicer (Fotodyne Inc., New Berlin, WI). Slices were explanted onto Millipore insert membranes, placed in six-well culture plates, and fed with regular slice culture medium (50% basal Eagle medium, 25% Earl’s balanced salt solution, 136 mM sodium chloride, 2 mM calcium chloride, 2.5 mM magnesium sulfate, 5 mM sodium bicarbonate, 3 mM glutamine, 40 mM glucose, 0.5 mM ascorbic acid, 20 mM HEPES buffer, 1 mg/L insulin, 5 U/ml penicillin and 5 mg/L streptomycin; pH 7.3) supplemented with 5% fetal bovine serum and 5% horse serum. Slices were kept in culture for 2 weeks and fed with 1 ml of fresh medium every other day. Slices were then treated with CX614, glutamate receptor antagonists, or calpain inhibitor III, at the following concentrations: CX614, 50 µM; AP-5, 100 µM; CNQX, 50 µM; calpain inhibitor III, 10 µM. At the indicated times after treatment initiation, slices were collected and processed for immunoblotting, immunocytochemistry, or in situ hybridization as described below. The AMPAr positive modulator CX614 was generously provided by Cortex Pharmaceuticals (Irvine, CA). AP-5, CNQX, and calpain inhibitor III were purchased from Sigma (St. Louis, MO).

Immunocytochemistry. Slices were washed twice in cold phosphate-buffered saline, fixed overnight with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at
4 °C, post-fixed and cryo-protected overnight at 4 °C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) supplemented with 20% sucrose, and then sectioned using a freezing microtome. Twenty-five μm-thick free-floating sections were washed in 0.1 M PB and blocked for 1 hr with 10% horse or goat serum in 0.1 M PB (+1% Triton X-100) and then incubated with the primary antibody (1:1000) overnight at 4 °C in 5% horse serum. Tissue was then washed in 0.1 M PB and incubated with the biotinylated secondary antibody (anti-rabbit; diluted 1:400) in 5% horse or goat serum/0.1 M PB for 2 h at room temperature. Following washes in 0.1 M PB, the tissue was incubated in Avidin Biotin Complex working solution (ABC Elite Vectastain kit; Vector Laboratories, Burlingame, CA) and then reacted using the Peroxidase Substrate SK-4100 Vector kit (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine as chromagen. Following final washes in 0.1 M PB, the sections were mounted onto Superfrost plus slides (Fisher Scientific, Tustin, CA), air-dried, dehydrated with ethanol, cleared with Americlear (Fisher Scientific, Tustin, CA) and coverslipped. Stained sections were examined using a Zeiss Axiskop2 microscope fitted with an AxioCam camera and operated with AxioVision 3.1.2.1 software (Carl Zeiss Vision, Thornwood, NY).

**Polyacrylamide gel electrophoresis and Western blotting.** Slices were collected into cold homogenizing buffer (50 mM Tris pH 7.5; 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM NaF, 1 mM Na3VO4; all from Sigma, St. Louis, MO) supplemented with protease inhibitor cocktail (Sigma) [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 2.08 mM; aprotinin, 1.6 μM; leupeptin, 40 μM; bestatin, 80 μM; pepstatin A, 30 μM; and trans-3-carboxyoxirane-2-carbonyl-L-leucylagmatine, 28 μM], sonicated 5 times using a Virsonic Cell Disruptor set at 50% for...
4-7 seconds intervals, and protein concentrations were determined. Equal amounts (20-40 µg) of sample proteins were separated according to their molecular weight on 8-10% sodium dodecyl sulfate (SDS) polyacrylamide gels and transferred onto nylon/PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Following transfer, membranes were blocked with 5% milk in 1x TBS-T (137 mM NaCl, 20 mM Tris base, 0.1% Tween 20; pH 7.4) for 1.5 h. Membranes were probed with the primary antibody diluted with 5% milk (1:1000) and incubated overnight at 4 °C. Membranes were then washed with 1x TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ) in a 1:10,000 dilution with 1x TBS-T for 1 h followed by washing in TBS-T. Membranes were exposed to ECL Western blotting detection reagent (Amersham) for 2 min, placed in an autoradiography cassette and exposed to Hyperfilm (Amersham) for different lengths of time. All membranes were stripped and re-probed with anti-actin antibodies (Sigma, St. Louis, MO) as loading controls. Intensities of immunoreactive bands were quantified by densitometry, normalized to actin content and analyzed by Student’s t-test.

**Antibodies.** For Western blotting, the following primary antibodies were used at the indicated final concentrations: monoclonal mouse anti-actin (0.1 µg/ml; Sigma, St. Louis, MO), polyclonal rabbit anti-GluR1 (0.5 µg/ml; Chemicon, Temecula, CA), polyclonal rabbit anti-GluR2/3 (0.5 µg/ml; Chemicon), polyclonal rabbit anti-NR1 (0.5 µg/ml; Chemicon) and polyclonal rabbit anti-NR2A/B (0.5 µg/ml; Chemicon), polyclonal rabbit anti-GRIP1 (Upstate Biotech, Chicago, IL), monoclonal mouse anti-SAP97 (Stressgen, Victoria, BC). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham (Piscataway, NJ) and used at 1:10,000 dilution. For
immunocytochemistry, the polyclonal anti-GluR1, anti-GluR2/3 antibodies, and a monoclonal mouse anti-GluR2 (Chemicon, Temecula, CA) antibody were used; All antibodies were diluted to 5-10 µg/ml. Secondary antibodies for immunocytochemistry were purchased from Vector Laboratories (Burlingame, CA).

**In situ hybridization.** Following drug treatments, cultures were washed twice with Tris-buffered saline and fixed overnight in cold fixative solution (4% paraformaldehyde solution in 0.1 M PB, pH 7.3). Cultures were kept in fixative at 4 ºC until sectioning at 25 µm with a freezing microtome. Sections were mounted onto Superfrost Plus slides and processed for the in situ hybridization localization of GluR1 and GluR2 mRNAs using ^35^S-labeled complementary RNA probes at a concentration of 1x10^7 cpm/ml with hybridization incubation times of 16-20 h at 60 ºC as previously described (Lauterborn et al., 2000). The GluR1 and GluR2 complementary RNAs were transcribed from BglI and BamHI digests of p59/2 and pRB14, respectively, and are complementary to 895 and 900 bases, respectively, of the 3′- ends including the non-coding region. Following hybridization, the tissue was treated with RNAse A (20 µg/ml, 30 min, 45 ºC) and washed in descending concentrations (2X - 0.1X) of saline sodium citrate buffer, 30 min each (1 X = 0.15 M NaCl / 0.015 M NaCitrate, pH 7.0). The tissue was air-dried and processed for Biomax film (Eastman Kodak, Rochester, NY) autoradiography with exposure times of 1-2 days. Hybridization densities within hippocampal CA1 and CA3b stratum pyramidale were measured from film autoradiograms, with labeling densities calibrated relative to film images of ^14^C-labeled standards (American Radiochemicals Inc., St. Louis, MO) using the AIS imaging system (Imaging Research Inc., St. Catherines, Ontario). For each field, significance of effect of
Lactate dehydrogenase activity. Lactate dehydrogenase (LDH) enzymatic activity in the culture medium was used to evaluate the extent of cellular damage produced in cultured slices subjected to the different treatments. Briefly, culture medium was collected following 8 or 48 h incubation of slices with CX614 alone, calpain inhibitor III alone, the glutamate receptor blockers AP-5 and CNQX, CX614 + glutamate receptor blockers, CX614 + calpain inhibitor III, and from untreated control cultures. Spectrophotometric determination was used to measure LDH activity by monitoring the rate at which the substrate, pyruvate, is reduced to lactate (Regan and Choi 1994). Reaction mixtures consisted of 600 µl of potassium phosphate buffer (0.1 M, pH 7.5 at 25 °C), 400 µl of supernatant culture medium and 500 µl of sodium pyruvate solution (22.7 mM in 0.1 M phosphate buffer, pH 7.5). The absorbance was read at 340 nm at 30 second intervals for 3 minutes. All reagents were purchased from Sigma (St. Louis, MO).
RESULTS

Treatment of cultured hippocampal slices with CX614 induces a gradual loss of AMPAR subunits. Hippocampal slices obtained from postnatal day 10 rat pups were maintained in culture for 10-14 d. Previous studies by our laboratories indicated that treatment of cultures for 48 h with CX614 decreased AMPAR subunit mRNA and protein levels (Lauterborn et al., 2003). In order to assess the time-course of CX614 on AMPAR protein, cultured slices were incubated with CX614 (50 µM) for 0, 4, 8, 12, 24 and 48 h (Fig. 1). Significant decreases in GluR1 and GluR2/3 levels were observed after 4 h of incubation. There was a near-linear decline in GluR1 and GluR2/3 subunits over time with CX614 treatment through 24 h (Fig. 1B). The decrease in AMPAR subunit levels continued at a slower rate between 24 and 48 h of treatment. Probing the same samples with antibodies specific to the NMDA receptor subunits NR1 and NR2A/B did not show significant differences from control at any time point (Fig. 1C); levels of NR1 subunits were decreased by ~ 15% while those of NR2A/B were increased by ~ 5% after 48 h treatment with CX614 (Fig. 1D). The results for GluR1 and GluR2/3 subunits were confirmed using immunohistochemistry; untreated control cultures and cultures treated with CX614 for 48 h were stained with the following AMPAR-specific antibodies: anti-C-terminal GluR1, anti-C-terminal GluR2/3 and anti-N-terminal GluR2 (Fig. 1E). Overall, immunostaining with all three antibodies was decreased. In addition, with antibodies against the C-terminal domain of GluR1, intense staining of some cell bodies was observed, an effect reminiscent of what we previously observed following calcium treatment of tissue sections (Bi et al., 1998).
Prolonged treatment of hippocampal slices with CX614 results in degradation of AMPA-associated PDZ proteins. AMPA receptor localization and trafficking in neurons are regulated by interactions of the receptors with several anchoring proteins, including SAP97 and GRIP1. Potential CX614-mediated degradation of SAP97 and GRIP1 could hinder trafficking of AMPArs to the cell membrane, destabilize the postsynaptic AMPAr compartment, and contribute to, or facilitate, CX614-induced loss of AMPAr subunits. To test for this possibility, levels of SAP97 and GRIP1 were assessed in hippocampal slices incubated for 48 h with or without CX614 (50 µM) (Fig. 2A). In Western blots, anti-SAP97 antibodies labeled three bands migrating at 140, 125 and 97 kDa, which were previously described as representing different SAP97 isoforms (Fig. 2A, upper panel). A comparable decrease in levels of the three bands occurred following CX614 treatment; in this and subsequent figures, levels of the major SAP97 band at 97 kDa was used as a representation of overall changes in SAP97 protein levels. CX614 treatment for 48 h decreased SAP97 levels to 44 ± 18% of control values (Fig. 2B). Similarly, GRIP1 levels were decreased to 58 ± 14% of control in CX614-treated samples (Fig. 2B). These results were confirmed using immunocytochemistry; GRIP1-immunoreactivity was uniformly reduced in CX614-treated slices as compared to untreated control slices (data not shown). Following prolonged exposure of Western blots to film, bands corresponding to native SAP97 and GRIP1 proteins were saturated (Fig. 2C). However, additional lower molecular weight bands became visible and these were more intense in CX614-treated as compared to control conditions (Fig. 2C), suggesting that CX614 treatment resulted in proteolysis of the native proteins. Quantification of the
intensity of these bands indicated that treatment of slices with CX614 increased levels of these fragments by 219 ± 9% for SAP97 and by 178 ± 9% for GRIP1 as compared to untreated control slices (Fig. 2D).

**Parallel effects of CX614 on AMPAr subunits and SAP97 and GRIP1.** Incubation of cultured hippocampal slices with CX614 induced a marked decline in AMPAr subunit levels within 8 h. To determine whether these effects of CX614 were reversible, cultures were treated with CX614 for 8 h, then transferred to control medium and maintained for various lengths of time (0, 4, 8, 12, 24, and 48 h). In parallel, sister cultures were exposed continuously to CX614 for 48 h. Cultures were collected and levels of AMPAr subunits, SAP97, and GRIP1 were analyzed by Western blotting. As expected, treatment of cultures with CX614 for 8 h induced a significant decrease in levels of AMPAr subunits and SAP97 (Fig. 3). Similarly, slices treated continuously with CX614 for 48 h exhibited a large decrease in levels of AMPAr subunits and of their partner PDZ proteins. Transfer of cultures to fresh CX614-free medium was not sufficient to immediately reverse the effects of the drug, as levels of AMPAr subunits, SAP97, and GRIP1 continued to decline for another 4-8 h. Levels of AMPAr subunits and of their partner PDZ proteins had slightly recovered by 24 h of drug wash-out. However, even following 48 h of incubation in the absence of CX614, levels of AMPAr subunits, SAP97 and GRIP1 did not completely return to control levels.

**Effects of CNQX and AP-5 on CX614-mediated decreases in AMPAr subunit and PDZ protein levels.** To investigate whether activation of AMPA and NMDA receptors
was involved in CX614-mediated down-regulation of AMPAr subunits and their PDZ partner proteins, control (DMSO vehicle) or CX614-treated hippocampal slice cultures were incubated with the NMDAr antagonist AP-5 (50 µM) and the AMPAr antagonist CNQX (100 µM). Cultured slices were treated with or without AP-5 + CNQX for 30 min before the addition of CX614 (50 µM) and then further incubated for 48 h. Levels of AMPAr subunits, SAP97, and GRIP1 were determined by Western blotting (Fig. 4A). Quantification of the blots (Fig. 4B) demonstrated that treatment of cultures with CNQX + AP-5 increased levels of GluR1 subunits without affecting levels of GluR2/3, SAP97 and GRIP1 (Fig. 4B). Furthermore, combined treatment with CNQX + AP-5 blocked the effects of CX614 on GluR1, GluR2/3, SAP97 and GRIP1 protein levels.

To assess whether CX614 effects on AMPAr and PDZ protein down-regulation involved activation of NMDAr and/or AMPAr, we incubated slices with CX614 alone, AP-5 alone, CNQX alone, CX614 + AP-5 (50 µM), or CX614 + CNQX (100 µM) (Fig. 4C). The results indicated that although CNQX was more efficacious than AP-5, it did not completely block CX614-induced decrease in GluR1 and SAP97 (Fig. 4D). Finally, treatment with AP-5 alone significantly reduced GluR1, GluR2/3, and GRIP1 levels as compared to untreated control slices (DMSO vehicle) but had no effect on SAP97 whereas treatment with CNQX alone had no significant effects on GluR1, GluR2/3 and GRIP1 but significantly reduced SAP97 expression.

Prolonged exposure of cultured hippocampal slices to CX614 decreases the expression of AMPAr subunit mRNAs. To further understand the mechanisms of CX614-mediated down-regulation of AMPA receptor function, we used in situ
hybridization to analyze the effects of prolonged treatment of cultured hippocampal slices with CX614 on GluR1 and GluR2 mRNA levels (Fig. 5). In a first set of experiments, cultured hippocampal slices were incubated under control conditions or with CX614, AP-5 + CNQX, or CX614 together with AP-5 + CNQX for 8 h. Although GluR1 mRNA levels showed a tendency to decrease in CX614-treated slices, no significant differences in GluR1 or GluR2 mRNA levels were observed under any conditions in CA1 and CA3 pyramidal cell layers at this time point (data not shown). In contrast, slices incubated for 48 h with CX614 exhibited a marked decrease in GluR1 and GluR2 mRNA levels in the pyramidal cell layer of both hippocampal subfields (Fig. 5). Treatment with CNQX + AP-5 alone for 48 h had opposite effects on GluR mRNA levels with modest increases in GluR1 (consistent with protein changes presented in Fig. 4) and small decreases in GluR2 mRNA levels: significant effects of treatment were reached for GluR1 and GluR2 mRNA levels in CA3 and CA1, respectively. Interestingly, treatment with CNQX + AP-5 markedly reversed the effects of CX614 for both mRNAs only in CA3. Two-way ANOVA analysis revealed a significant interaction between CX614 and the blockers for GluR2 mRNA (p = 0.0006) and a strong trend for GluR1 mRNA (p = 0.0509). By contrast, in region CA1, CNQX + AP-5 treatment did not block the CX614-induced decreases in GluR mRNA levels. Two-way ANOVA analysis revealed no significant interaction between CX614 and the blockers for both GluR1 (p = 0.008) and GluR2 (p = 0.0075) mRNAs in this field.

Effects of a calpain inhibitor on CX614-induced down-regulation of AMPA receptors and their interacting PDZ proteins. As mentioned above, treatment of cultures with CX614
increased the amount of small molecular weight bands that reacted with SAP97 and GRIP1 antibodies. We previously identified GRIP1 and PSD-95, another PDZ protein that shares a similar structure with SAP97 and interacts with glutamate receptors, as substrates of the calcium-activated protease calpain (Lu et al., 2000, 2001). Preliminary results showed that probing brain membrane fractions pre-treated with Ca++ (2 mM; 30 min at 37 ºC, conditions that activate endogenous calpain) with anti-GRIP1 antibodies showed bands that migrated at the same level as those generated following treatment of cultured hippocampal slices with CX614 (data not shown). Moreover, we recently reported that CX614 treatment activates calpain and leads to the degradation of the cytoskeletal protein spectrin (Jourdi et al., 2005). These results suggested that prolonged treatment with CX614, could result in calpain-mediated SAP97 and GRIP1 degradation, thereby leading to AMPAr down-regulation by interfering with PDZ protein-dependent stabilization and/or intracellular AMPAr trafficking. To test this hypothesis, we incubated cultured hippocampal slices with CX614 with or without calpain inhibitor III for 48 h. Western blot analysis indicated that calpain inhibitor III significantly reduced CX614-induced decreases in AMPAr subunits as well as decreases in the levels of their interacting PDZ proteins. Again, the results underscored the parallel effects of calpain inhibition on GluR1 and SAP97 and on GluR2/3 and GRIP1. In particular, calpain inhibitor III almost completely reversed the effect of CX614 on GRIP1 and GluR2/3. We also tested whether calpain inhibitor III could block the effects of CX614 on the levels of GluR1 and GluR2 mRNA (Fig. 7). Incubation of slices with calpain inhibitor III alone for 48 h had no significant effect on GluR1 and GluR2 mRNA levels in any subfield of hippocampus. However, unlike the protein effects, calpain inhibitor III had no significant
effect on CX614-induced decreases in levels of GluR1 and GluR2 mRNAs. Two-way ANOVA indicated no significant effect of calpain inhibitor III on CX614-induced decrease in GluR mRNA levels in either hippocampal subfield.

**CX614 effects are not due to excitotoxicity.** Lactate dehydrogenase (LDH) activity has been widely used to assess the integrity of the cell membrane and as a measure of toxicity-induced cellular damage that could lead to cell death (Regan and Choi 1994). In order to investigate whether the marked decreases in levels and expression of AMPAR subunits and PDZ proteins following prolonged incubation of cultured hippocampal slices with CX614 could result from neuronal toxicity, we measured LDH activity in the culture medium from hippocampal slices treated with the AMPAR modulator under a variety of experimental conditions for 8 h and 48 h (Fig. 8). There were no significant differences in LDH activity in cultures treated with CX614 alone, calpain inhibitor III alone, or CNQX + AP-5 at either time point. Similarly, treatment of slices with combinations of CX614 and calpain inhibitor III, or CX614 and AP-5 + CNQX did not modify LDH activity.
DISCUSSION

Previous studies have shown that treatment with CX614 in cultured hippocampal slices over several hours increases AMPAr-mediated currents. However, with longer exposure intervals, CX614 decreases AMPAr currents, suggesting either decreased receptor responsiveness, increased receptor desensitization or loss of AMPAr expression over time (Arai et al., 2000, 2002a; Lauterborn et al., 2000, 2003; Nagarajan et al., 2001). The present data demonstrate that indeed there is a loss of AMPAr mRNA and protein expression following chronic CX614 treatment. Sustained CX614-induced increases in glutamatergic transmission could over time become toxic and such toxicity might provide an explanation for CX614-induced decrements in AMPAr-mediated currents. Our results reveal that despite its ability to down-regulate AMPAr protein levels and their mRNAs, CX614 did not increase LDH release, arguing against toxicity and implying uncompromised cell membrane integrity. Also, the present AMPAr immunocytochemical results and previous morphological evaluation of various hippocampal subfields in Nissl-stained tissue sections did not reveal any signs of cell death in ampakine-treated slice cultures (Lauterborn et al., 2000). In addition, CX614 is a potent inducer of the neuroprotective and survival-promoting neurotrophin BDNF (Lauterborn et al., 2000).

Recently, we showed a gradual accumulation of CX614-induced calpain-mediated spectrin breakdown products that was completely blocked by calpain inhibitor III and by the AMPAr blocker CNQX but unaffected by the NMDAr blocker AP-5 (Jourdi et al., 2005). In the present study, decreases in levels of AMPAr subunits and of their respective anchoring PDZ proteins were significant after 4 h of CX614 treatment and became
greater with longer incubation intervals. Co-administration of AP-5 and CNQX completely blocked CX614 effects on AMPAr subunits and PDZ proteins. The effects of CX614 on AMPAr and PDZ proteins were partially reduced in the presence of CNQX alone and AP-5 was less efficacious in blocking CX614 effects on AMPArs and PDZ proteins. Previous studies have demonstrated that NMDArs are tonically active in hippocampal slices (Sah et al., 1989), and our results indicated that AP-5 partially reduced CX614 effects on SAP97 and GluR2/3. In addition, AP-5 significantly decreased CX614 effects on GRIP1 but not on GluR1. Taken together, our results argue against NMDArs being the principal source of increases in intracellular Ca^{++} needed for calpain activation with the manipulations described here. However, AMPArs are Na^{+} channels and are, by and large, impermeable to Ca^{++} ions (Lomeli et al., 1994; Jensen et al., 1998; Carlson et al., 2000; Iizuka et al., 2000; Krampfl et al., 2002; Kumar et al., 2002). Interestingly, elevated intracellular Na^{+} levels can increase Ca^{++} release from intracellular stores (Hoyt et al., 1998; Zhang and Lipton 1999), thus CX614-induced increases in AMPAr function and the resulting depolarization may contribute to increased intracellular levels of Ca^{++}. Pertinent to this, ryanodine receptors, which regulate intracellular Ca^{++} release, are also targets for calpain proteolysis (Shoshan-Barmatz et al., 1994). Thus, CX614-dependent increases in AMPAr activity might enhance intracellular Ca^{++} concentrations and consequently activate calpain which then can truncate ryanodine receptors; this would exacerbate Ca^{++} release from intracellular stores and further enhance calpain-dependent degradation of AMPAr-interacting PDZ proteins and spectrin. Interestingly, calpain activation, ryanodine receptors, and the release of Ca^{++} from intracellular stores are tied to aspects of AMPAr function. Calpain activation is required
for LTP induction (Vanderklish et al., 1996) and recent studies of type 3 ryanodine receptors provide strong support for the involvement of intracellular Ca\(^{++}\) stores in calpain activation as deletion of this receptor impairs some forms of AMPAr-mediated synaptic plasticity, LTP and spatial learning (Balschun et al., 1999; Shimuta et al., 2001).

Our results clearly show that calpain activation is predominantly downstream from AMPArs and that it mediates CX614-induced AMPAr protein loss and degradation of PDZ proteins and spectrin (Jourdi et al., 2005). In particular, calpain inhibition blocked CX614-induced decrease in PDZ protein levels and formation of PDZ protein degradation products, and blocked CX614 effects on AMPAr subunits. In addition, down-regulation of individual AMPAr subunits and their recovery after CX614 wash-out were associated with similar changes in their interacting PDZ proteins. GRIP1 is implicated in GluR2 stabilization (Jourdi et al., 2003) and GRIP1 truncation by calpain has been shown to decrease GRIP1-GluR2 association (Lu et al., 2001). Our data also indicate that SAP97, which is implicated in GluR1 intracellular trafficking, its insertion in membranes and in the stabilization of GluR1 protein (Jourdi et al., 2003; Collingridge et al., 2004) is a calpain substrate as well.

Calpain activation leads to the truncation of GluR1 and GluR2 C-terminal domains (Bi et al., 1998). Thus, a reduction in AMPAr subunits and/or a loss of their C-terminal epitopes could contribute to CX614-induced decreases in AMPAr immunoreactivities observed in this study. Although our immunostaining results cannot exclude truncation of AMPAr C-terminal domains from being responsible for lower levels of immunoreactivity, the results obtained with the anti-N-terminal GluR2 antibody strongly favor the loss of total protein expression with chronic exposure to CX614, at
least for GluR2. In addition, truncation of GluR1 and GluR2 C-terminal domains (Bi et al., 1998) and degradation of PDZ proteins and spectrin by calpain might dissociate AMPArs from the cytoskeleton, change their channel properties and alter their rate of internalization.

AMPAr down-regulation generally involves dissociation from interactions with cytoskeletal and PDZ proteins and is followed by internalization and proteolysis (Ahmadian et al., 2004; Collingridge et al., 2004; Lee et al., 2004). As such, prolonged application of CX614 is likely to cause destabilization and internalization of surface-bound receptors and reduced recycling to the synapse given the down-regulation of SAP97 and GRIP1 and their calpain-mediated degradation observed here. In fact, the interaction of AMPArs with the cytoskeleton and their internalization are highly regulated mechanisms involving several kinases and phosphatases (Rong et al., 2001; Iwakura et al., 2001; Ahmadian et al., 2004; Collingridge et al., 2004). Future studies will investigate whether CX614, and other positive AMPAr modulators, recruit distinct kinase pathways to regulate AMPAr surface expression. In addition, various manipulations can induce AMPAr internalization including LTD or treatment with insulin. The type and duration of the inducing stimulus determine the fate of internalized AMPArs that can be either recycled to the synapse or targeted for terminal proteolysis (Iwakura et al., 2001; Ahmadian et al., 2004; Collingridge et al., 2004; Lee at al., 2004). Future studies will investigate whether LTD-induced AMPAr internalization implicates calpain and whether CX614, and other positive AMPAr modulators, regulate AMPAr surface expression.

BDNF is important for AMPAr expression (Narisawa-Saito et al., 2002; Jourdi et al., 2003) and chronic exposure of hippocampal slice cultures to CX614 initially leads to
increases in BDNF mRNA and protein expression that are not maintained past 24-48h. However, intermittent application of CX614 was successfully applied and resulted in sustained increases in BDNF mRNA and protein levels (Lauterborn et al., 2003). Thus, future studies will assess the effects of similar protocols on AMPAr expression, PDZ protein degradation and calpain activation.

Consistent with the protein data, prolonged exposure to CX614 also affected AMPAr gene expression and reduced their mRNA levels. The loss of GluR mRNA expression occurred within all principal cell layers of hippocampus with 48h CX614 treatment. While the combination of AP-5 and CNQX significantly inhibited CX614-induced decreases in total hippocampal AMPAr protein levels, these blockers did not equally inhibit the decrease in GluR mRNA levels between regions CA3 and CA1. AP-5 and CNQX co-treatment blocked the reduction in GluR mRNA levels in CA3, but was ineffective in CA1. One explanation for this regional disparity may be due to developmental differences in AMPA receptor antagonist binding between the CA3 and CA1 subfields. During postnatal development ³H-CNQX binding attains adult levels in CA3 by postnatal day 15 whereas in CA1 it is not attained until postnatal day 40 (Standley et al., 1995). Given that the present study utilized slice cultures that at the time of drug treatment approximated postnatal day 20 animals, the deficiency in CA1 ³H-CNQX binding may account for the regional difference in the blocking effect observed here. Alternatively, the differential effects of the combination CNQX and AP-5 on CX614-induced decreases in GluR mRNA levels between the hippocampal subfield might reflect greater spontaneous release of glutamate in CA1 than in CA3.
Overall, the main findings suggest that several factors can contribute to CX614-induced down-regulation of AMPArs. First, enhancing AMPAr activity is a major mechanism underlying AMPAr down-regulation; it involves activity-dependent, calpain-mediated degradation of spectrin and AMPAr-interacting PDZ proteins, which would be expected to interfere with receptor stabilization and trafficking. A second mechanism of AMPAr down-regulation involves calpain-independent decrease of the transcription of the genes coding for these subunits, at least in CA1. Future studies should assess whether other manipulations which increase synaptic activity in cultured slices (e.g., treatment with functionally distinct ampakines, inhibition of GABAergic transmission) have similar or differential effects on levels of PDZ proteins and AMPArs to those observed here.

In conclusion, this study further implicates calpain in physiological activity-induced changes in postsynaptic structures in the absence of cell toxicity and demonstrates that with prolonged enhancement of glutamatergic synaptic activity AMPAr loss 1) is mediated by increased AMPAr activation, 2) involves calpain-dependent proteolysis of SAP97 and GRIP1, 3) is reflected at the mRNA level by suppression of AMPAr gene expression, and 4) is not associated with cell damage.
ACKNOWLEDGMENTS

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REFERENCES


FOOTNOTES

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Legends for Figures

Fig. 1. CX614-induced down-regulation of AMPAr in cultured hippocampal slices. Cultures were incubated in the absence (DMSO vehicle control) or presence of 50 µM CX614, and processed for immunoblotting or for immunostaining. A) Representative blots of samples from cultures incubated continuously for 48 h with the vehicle control or with 50 µM CX614 for 4, 8, 24 or 48 h labeled with anti-GluR1 or anti-GluR2/3 antibodies. B) Quantification of the levels of GluR1 and GluR2/3 from 4 duplicate experiments (total n = 8 samples per time point). *, *: p <0.05; **: p <0.01; *** p <0.001, versus control levels, Student's t-test. C) Representative blots of samples from control cultures or cultures treated with 50 µM CX614 for 48 h labeled with anti-NR1 or anti-NR2A/B antibodies. D) Quantification of results derived from 4 duplicate experiments did not show significant differences in NR1 and NR2A/B protein levels compared to controls. Dashed line indicates protein expression in vehicle control samples. E) Photomicrographs showing immunostaining with AMPAr-specific antibodies in region CA1 of cultured hippocampal slices either untreated (left column) or treated with CX614 for 48 h (right column). Sections were incubated with anti-C-terminal GluR1, anti-C-terminal GluR2/3 or anti-N-terminal GluR2 antibodies. Scale bar = 100 µM.

Fig. 2. Effects of CX614 on AMPAr-associated PDZ proteins. Cultured hippocampal slices were incubated with 50 µM CX614 or vehicle (control) for 48 h and processed for immunoblotting with SAP97 and GRIP1 antibodies. A) Representative blots for SAP97 and GRIP1. B) Quantification of major band densities obtained in 4 duplicate experiments.
experiments (total n = 8 samples / group) for SAP97 and GRIP1. Dashed line indicates protein expression in vehicle control samples. C) Representative immunoblots showing increased intensities of lower molecular weight bands reacting with SAP97 and GRIP1 antibodies in CX614-treated samples. D) Quantification of lower molecular weight species (arrows) from 8 experiments for SAP97 and GRIP1 degradation products. Dashed line indicates protein expression in vehicle control samples. *: $p < 0.01; **: p < 0.005$, Student’s $t$-test.

**Fig. 3.** Time-course for the recovery of AMPAr and PDZ protein expression. Bar graphs showing protein levels for GluR1 and SAP97 (A) and GluR2/3 and GRIP1 (B) in cultured hippocampal slices that were either treated with 50 µM CX614 for 8 h and then transferred to CX614-free fresh culture medium (wash) and collected various times thereafter (open symbols) or treated continuously with CX614 for 48 h (solid symbols); data are expressed as percent of vehicle-control levels and are means ± S.E.M. of 3 duplicate experiments. *: $p < 0.05; **: p < 0.005; \ddagger: p < 0.05; \ddagger\ddagger: p < 0.01$, Student’s $t$-test.

**Fig. 4.** Effects of CNQX and AP-5 on CX614-induced changes in levels of AMPAr subunits and their associated PDZ proteins. Hippocampal slice cultures were incubated with or without 50 µM CX614 for 48 h, and in the presence or absence of AP-5 (100 µM) and CNQX (50 µM). A,C) Representative western blots labeled with anti-GluR1, -GluR2/3, -SAP97 or -GRIP1 antibodies under various conditions examining the effects of either co-application of AP-5 and CNQX (A) or separate application of the blockers (C) on CX614 effects. B) Quantification of 4 duplicate experiments as shown in Panel A with
results expressed as percent of control values. D) Quantification of 4 duplicate experiments as shown in Panel C with results expressed as percent of control values. *: $p < 0.05$; **: $p < 0.01$; ‡: $p < 0.05$; ‡‡: $p < 0.01$, Student's $t$-test; $n = 8$ per group.

**Fig. 5.** Effects of CNQX and AP-5 on CX614-induced down-regulation of GluR1 and GluR2 mRNA levels. A) Photomicrographs of film autoradiograms showing in situ hybridization for GluR1 mRNA in cultured hippocampal slices under control conditions (vehicle) or treated for 48 h with CX614 alone, CNQX + AP-5, or CX614 + CNQX + AP-5 (Top). Bar graphs showing quantification of GluR1 cRNA hybridization levels in the pyramidal cell layer of regions CA3 and CA1 obtained in 3 duplicate experiments ($n = 9$-$14$ cultures/group). B) Photomicrographs of film autoradiograms showing in situ hybridization for GluR2 mRNA in cultured hippocampal slices under control conditions or treated for 48 h with CX614 alone, CNQX + AP-5, or CX614 + CNQX + AP-5 (Top). Bar graphs showing quantification of GluR2 cRNA hybridization levels in the pyramidal cell layer of regions CA3 and CA1 obtained in 3 duplicate experiments ($n = 14$-$15$ cultures/group). *: $p < 0.01$; **: $p < 0.05$; ***: $p < 0.005$ Student-Newman-Keuls; scale bar = 500 µm.

**Fig. 6.** Effects of calpain inhibitor III on CX614-mediated down-regulation of AMPAR subunits and their associated PDZ proteins. A) Representative immunoblots of samples probed for the total protein expression of GluR1, GluR2/3, SAP97 and GRIP1 in cultured hippocampal slices treated with CX614 (50 µM) with or without calpain inhibitor III (Cal Inh III; 10 µM) for 48 h. Samples were obtained from slices treated in the absence of
CX614 and calpain inhibitor III (Control), CX614 alone, calpain inhibitor III alone (Cal Inh III), or the combination CX614 + Cal Inh III. B) Quantification of the immunoreactive bands obtained in 4 duplicate experiments (total n = 8/group); results are expressed as percent of the values in control conditions and are means ± S.E.M. Dashed line indicates protein expression levels in control samples. *: $p < 0.01$; **: $p < 0.05$, Student's $t$-test.

**Fig. 7.** Effects of calpain inhibitor III on CX614-induced decrease in AMPAr subunit mRNA levels. Control and CX614-treated (50 µM) cultured hippocampal slices were incubated with and without calpain inhibitor III (Cal Inh III; 10 µM) for 48 h. A) Photomicrographs showing the in situ hybridization localization of GluR1 mRNA in cultured hippocampal slices under control conditions, or treated for 48 h with CX614 alone, calpain inhibitor III (Cal Inh III) alone, or CX614 + Cal Inh III (Top). Bar graphs show quantification of film autoradiograms for GluR1 cRNA densities obtained in 3 duplicate experiments (n = 12-18 cultures/group). B) Photomicrographs showing the in situ hybridization localization of GluR 2 mRNA in cultured hippocampal slices under control conditions, or treated 48 h with CX614 alone, calpain inhibitor III (Cal Inh III) alone, or CX614 + Cal Inh III (Top). Bar graphs shows quantification film autoradiograms for GluR2 cRNA densities obtained in 3 duplicate experiments (n = 15-18 cultures/group). *: $p < 0.005$, Student's $t$-test; scale bar = 500 µm.

**Fig. 8.** Effects of various prolonged treatment with CX614 and other agents on LDH release in cultured hippocampal slices. Slices were treated with CX614, CX614 + AP-5 +
CNQX, calpain inhibitor III, CX614 + calpain inhibitor III or left untreated. Incubation medium was collected following 8 h or 48 h incubation in the above-mentioned conditions, and assayed for lactate dehydrogenase (LDH) activity. Results are expressed as percent of control values and are means ± S.E.M. of 4 triplicate (n =12) experiments.
Figure 1

A)  

B)  

C)  

D)  

E)  

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Figure 2

A) 

B) 

C) 

D)
Figure 3

A)

CX614

Wash

% of Control Protein Expression

0 8 12 16 20 32 48 56 h

CX614

Δ GluR1

SAP97

B)

CX614

Wash

% of Control Protein Expression

0 8 12 16 20 32 48 56 h

Δ GRIP1

GluR2/3
Figure 4

A) Western blots showing protein expression levels for GluR1, GluR2/3, SAP97, and GRIP1 under different conditions: Control, CX614, CNQX + AP-5, and CNQX + AP-5 + CX614.

B) Bar graph depicting the percentage of control protein expression for GluR1, GluR2/3, SAP97, and GRIP1 under the same conditions as in A. The relative expression levels are compared visually and statistically.

C) Additional Western blots showing protein expression levels for GluR1, GluR2/3, SAP97, and GRIP1 under different conditions: Control, CX614, AP-5, CX614 + AP-5, CNQX, and CX614 + CNQX.

D) Bar graph showing the percentage of control protein expression for GluR1, GluR2/3, SAP97, and GRIP1 under the conditions as in C. The expression levels are compared and statistically analyzed.
Figure 5

A) GluR1 mRNA

B) GluR2 mRNA
Figure 6

A) Western blot analysis of GluR1, GluR2/3, SAP97, and GRIP1 with control, CX614, Cal Inh III, and CX614 + Cal Inh III.

B) Quantification of GluR1, GluR2/3, SAP97, and GRIP1 expression as a percentage of control. Significant differences are marked with * (p < 0.05) and ** (p < 0.01).
Figure 7

A) GluR1 mRNA

CA3

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CA1

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B) GluR2 mRNA

CA3

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Figure 8

A) Control CX614 AP-5 + CNQX CX614 + AP-5 + CNQX

% of Control Lactate Dehydrogenase Activity

8h 48h

B) Control Cal Inh III CX614 Cal Inh III CX614 + Cal Inh III

% of Control Lactate Dehydrogenase Activity

8h 48h