Evidence of calcium-permeable AMPA receptors in dendritic spines of CA1 pyramidal neurons

Hayley A. Mattison,1,2 Ashish A. Bagal,1 Michael Mohammadi,1 Nisha S. Pulimood,1,3 Christian G. Reich,1 Bradley E. Alger,1,2 Joseph P. Y. Kao,1,2,4 and Scott M. Thompson1,2

1Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland; 2Membrane Biology Training Program, University of Maryland School of Medicine, Baltimore, Maryland; 3Program in Neuroscience, University of Maryland School of Medicine, Baltimore, Maryland; and 4Center for Biomedical Engineering and Technology, University of Maryland School of Medicine, Baltimore, Maryland

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Mattison HA, Bagal AA, Mohammadi M, Pulimood NS, Reich CG, Alger BE, Kao JP, Thompson SM. Evidence of calcium-permeable AMPA receptors in dendritic spines of CA1 pyramidal neurons. J Neurophysiol 112: 263–275, 2014. First published April 23, 2014; doi:10.1152/jn.00578.2013.—GluA2-lacking, calcium-permeable α-amino-3-hydroxy-5-methylisoxazole-4-propionate receptors (AMPARs) have unique properties, but their presence at excitatory synapses in pyramidal cells is controversial. We have tested certain predictions of the model that such receptors are present in CA1 cells and show here that the polyamine spermine, but not philanthoxin, causes use-dependent inhibition of synaptically evoked excitatory responses in stratum radiatum, but not stratum oriens, in cultured and acute hippocampal slices. Stimulation of single dendritic spines by photolytic release of caged glutamate induced an N-methyl-D-aspartate receptor-independent, use- and spermine-sensitive calcium influx only at apical spines in cultured slices. Bath application of spermine also triggered a spermine-sensitive influx of cobalt into CA1 cell dendrites in stratum radiatum. Responses of single apical, but not basal, spines to photostimulation displayed prominent paired-pulse facilitation (PPF) consistent with use-dependent relief of cytoplasmic polyamine block. Responses at apical dendrites were diminished, and PPF was increased, by spermine. Intracellular application of pep2m, which inhibits recycling of GluA2-containing AMPARs, reduced apical spine responses and increased PPF. We conclude that some calcium-permeable, polyamine-sensitive AMPARs, perhaps lacking GluA2 subunits, are present at synapses on apical dendrites of CA1 pyramidal cells, which may allow distinct forms of synaptic plasticity and computation at different sets of excitatory inputs.

FAST EXCITATORY SYNAPTIC TRANSMISSION in the central nervous system is mediated by glutamate receptors having high affinity for α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA). AMPA receptors (AMPARs) are encoded by four genes, GluA1–4 (Collingridge et al. 2009; Hollmann and Heinemann 1994; Seeburg 1993). In the hippocampus, mature CA1 pyramidal cells express GluA1–3 (Gerfin-Moser et al. 1995; Keinanen et al. 1990). mRNA transcripts for GluA2 are edited twice in close succession gives rise to a polyamine block. Conversely, polyamine block of open channels increases with depolarization, giving rise to the inward rectification of GluA2-lacking AMPARs (Bowie and Mayer 1995; Donevan and Rogawski 1995; Kamboj et al. 1995; Koh et al. 1995). Because of their Ca2+-permeability and voltage-dependent signaling, GluA2-lacking AMPARs have been implicated in several forms of synaptic plasticity.

The subunit composition of synaptic AMPARs is difficult to determine directly. Immunoprecipitation suggests that 90% of hippocampal GluA1 subunits are bound to a GluA2 subunit (Wenthold et al. 1996). Indeed, extrasynaptic AMPARs in excised patches from CA1 pyramidal cell somata display linear current-voltage relationships (I–V; Jonas and Sakmann 1992), as do multisyapse evoked excitatory postsynaptic currents (EPSCs) in CA1 cells in acute rat hippocampal slices (Hestrin et al. 1990), albeit without the replacement of endogenous polyamines. These data have led to the tenet that all pyramidal cell synaptic AMPARs are Ca2+-impermeable and are either GluA1-GluA2 or GluA2-GluA3 heteromers. However, other studies have suggested that some pyramidal cell AMPARs are Ca2+-permeable (Lerma et al. 1994; Noh et al. 2005; Ogoshi and Weiss 2003; Rozov et al. 2012; Tsubokawa et al. 1995) and that individual synapses may express both Ca2+-permeable and -impermeable AMPARs (He et al. 2009; Isaac et al. 1995; Washburn et al. 1997). Lu et al. (2009) have established that homomeric GluA1 receptors can be formed in CA1 cells and trafficked to synapses. Toomim and Millington (1998) have permeable and sensitive to block by polyamines, although Ca2+-impermeable AMPARs containing GluA2 subunits have been described (Meucci and Miller 1998). Whether Ca2+-permeable, polyamine-sensitive AMPARs are present at excitatory synapses in the hippocampus or, alternatively, whether all synaptic AMPARs are Ca2+-impermeable has important consequences for the properties of the synapses and has been the subject of much recent debate.

The lack of positive charge in the pore of GluA2-lacking AMPARs allows positively charged polyamines to block the channel from inside or outside in a use- and voltage-dependent manner (Washburn and Dingledine 1996) whether the channel is open or closed. Closed channel blockade is relieved by cation influx (Bowie et al. 1998). Therefore, activating GluA2-lacking AMPARs twice in close succession gives rise to a postsynaptically mediated paired-pulse facilitation (PPF; Rozov et al. 1998; Rozov and Burnasheff 1999) when the current triggered by the first stimulus produces use-dependent relief of polyamine block. Conversely, polyamine block of open channels increases with depolarization, giving rise to the inward rectification of GluA2-lacking AMPARs (Bowie and Mayer 1995; Donevan and Rogawski 1995; Kamboj et al. 1995; Koh et al. 1995). Because of their Ca2+-permeability and voltage-dependent signaling, GluA2-lacking AMPARs have been implicated in several forms of synaptic plasticity.

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suggested that there may be a distinct localization of Ca\(^{2+}\)-permeable AMPARs in apical dendrites in area CA1.

Using extracellularly applied polyamines and focal photolysis of caged glutamate (Bagal et al. 2005), we find that Ca\(^{2+}\)-permeable AMPARs, perhaps lacking GluA2 subunits, may contribute as much as half of the current in single spines of apical, but not basal, dendrites of CA1 pyramidal neurons.

**MATERIALS AND METHODS**

**Hippocampal slice and tissue culture preparation.** Organotypic hippocampal slice cultures were prepared using the roller tube method (Gähwiler et al. 1998). In brief, 400-μm-thick hippocampal slices were prepared from 5- to 8-day-old rat pups and attached to polylysine-coated glass coverslips in a film of clotted chicken plasma (Cocalico Biologicals, Reamstown, PA) supplemented with fibrin (Tissel VH; Baxter Healthcare, Westlake Village, CA). Cultures were then maintained on a roller drum in horse serum-containing medium in an incubator for 14–28 days to allow for synaptic maturation. Hippocampal slices were also prepared acutely from adult male Sprague-Dawley rats using standard techniques. Rats were deeply anesthetized with urethane (2% in O\(_2\)) or isoflurane and decapitated. Transverse slices were cut at 400-μm thickness on a vibratome. Slices were held at room temperature in a holding chamber with humidified 95% O\(_2\)-5% CO\(_2\) for >1 h before recording. These protocols were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee.

**Electrophysiology.** Hippocampal slice cultures were transferred to a recording chamber mounted on the stage of an upright microscope (Nikon) equipped with differential interference contrast (DIC) optics. The chamber was perfused with saline containing, in mM, 137 NaCl, 2.8 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 11.6 NaHCO\(_3\), 2 HEPES, 0.4 NaH\(_2\)PO\(_4\), and 1.25 Ca\(^{2+}\). The perfusion solution was replaced with Oregon Green-2′,7′-dichlorodihydrofluorescein diacetate (OGB-2′,7′-DCHF-DA), or Alexa Fluor 488 dyes. As reported previously (Bagal et al. 2005), this protocol produces UV illumination at depth in the culture within a spot having a diameter at half-maximal amplitude of ~1 μm, close to the observed diffraction limit. Fluorescence emission was imaged using a charge-coupled device (CCD) camera (Orca ER II; Hamamatsu) effective pixel size = 0.012 μm\(^2\). Image acquisition was controlled by SimplePCI software (Hamamatsu).

**Cobalt loading.** Permeability of AMPA receptors to divalent cations was examined using the Co\(^{2+}\) technique, as described previously (Aurousseau et al. 2012). In brief, after recovering from slicing for 1 h in normal extracellular saline, 400-μm-thick hippocampal slices were placed in mesh-bottom cups and submerged in a series of solutions titrated to pH 7.4 by bubbling with 95% O\(_2\)-5% CO\(_2\) at room temperature. Slices were then equilibrated in assay buffer [57.5 mM NaCl, 5 mM KCl, 20 mM NaHCO\(_3\), 12 mM D(+)-glucose, 139 mM sucrose, 0.75 mM CaCl\(_2\), 2 mM MgCl\(_2\), 30 μM cyclothiazide (CTZ), and 0.01 mM phenol red] for 20 min. The cup containing the slices was then transferred to assay buffer containing 5 mM CoCl\(_2\) and 10 mM glutamate for 20 s to activate AMPARs followed by 40 s in assay buffer containing Co\(^{2+}\) alone. This 20-s Glu-on-40-s Glu-off protocol was repeated 10 times over 10 min to minimize desensitization of AMPARs. The slices were then transferred to assay buffer containing 2 mM EDTA for 5 min to chelate the extracellular Co\(^{2+}\) and then washed in assay buffer for 5 min. Finally, the slices were treated with 0.24% (NH\(_4\))\(_2\)S in assay buffer to precipitate intracellular Co\(^{2+}\) and washed again in assay buffer before being stored in 4% paraformaldehyde in 0.1% PBS at 4°C overnight.

Each 400-μm slice was embedded in a small cube of 10% gelatin in 0.1 M PBS and resectioned on a vibratome into 50-μm slices, which were then subjected to 60 min of silver intensification (GE Healthcare IntenSE protocol was repeated 10 times over 10 min to minimize desensitization of AMPARs. The slices were then transferred to assay buffer containing 2 mM EDTA for 5 min to chelate the extracellular Co\(^{2+}\) and then washed in assay buffer for 5 min. Finally, the slices were treated with 0.24% (NH\(_4\))\(_2\)S in assay buffer to precipitate intracellular Co\(^{2+}\) and washed again in assay buffer before being stored in 4% paraformaldehyde in 0.1% PBS at 4°C overnight.

Each 400-μm slice was embedded in a small cube of 10% gelatin in 0.1 M PBS and resectioned on a vibratome into 50-μm slices, which were then subjected to 60 min of silver intensification (GE Healthcare IntenSE M Silver Enhancement kit) to enhance Co\(^{2+}\) visualization. After incubation, the slices were washed thoroughly in water to arrest the action of the silver reagents and then imaged with DIC microscopy using a CCD camera (Orca ER II) and SimplePCI software.

**Calcium imaging.** To calculate the ΔF/ΔF values, the baseline fluorescence emission intensity (F) was calculated from background-subtracted gray values of circular regions of interest (ROIs) surrounding the spine head and averaged over 10–25 continuously acquired image frames (~25 ms frame-to-frame interval) corresponding to 500 ms preceding the uncaging stimulus. This baseline average was then subtracted from each gray value of the spine head before and after the uncaging stimulus to calculate the ΔF. Background subtraction was achieved by measuring gray values in ROIs adjacent to the stimulated spine head over 10–25 frames. This value was then subtracted from the gray value of the dendritic spine head for each image frame.

Integrals of the ΔF/ΔF signal traces were calculated by computing the three-point average of the ΔF/ΔF values and multiplying the three-point average by the frame-to-frame interval. The sum of the product was taken over 500 ms, or 10–25 values, following the uncaging stimulus. Signals were excluded from analysis if the time from the beginning of the signal (i.e., when ΔF/ΔF values first became >0.05) to end of the signal (when ΔF/ΔF values < 0.05) was not ≥350 ms and the signal-to-noise ratio was not ≥2. The signal-to-noise ratio was
calculated by dividing the root mean square of the \( \Delta F/F \) values over 100 ms (signal) following the uncaging stimulus by the root mean square of the \( \Delta F/F \) values over 500 ms preceding the uncaging stimulus (noise).

All data are presented as means ± SE. Significance values were determined using paired and unpaired, two-tailed \( t \)-tests as appropriate, unless the requirements for performing parametric tests were not met in the data set. In these cases, nonparametric tests were used, as noted in the text. Spine volume was calculated by tracing spine heads manually, measuring their area with SimplePCI software, and converting this measurement to volume, assuming spherical dimensions. All chemicals were obtained from Sigma (St. Louis, MO), Tocris (Ballwin, MO), or Invitrogen.

RESULTS

Synaptic responses are sensitive to the polyamine spermine but not philanthotoxin. Previous studies reported that the polyamines philanthotoxin-433 (PhTx) and 1-naphthyl-acetyl-spermine (NASPM) have little or no effect on EPSCs in area CA1 (Adesnik and Nicoll 2007; Moulit et al. 2010; Noh et al. 2005; Plant et al. 2006; Rozov et al. 2012). We repeated these experiments in acute hippocampal slices from adult (>250 g) rats with extracellular recording electrodes in stratum radiatum. Because N-methyl-D-aspartate receptors (NMDARs) are also sensitive to polyamines (Rock and Macdonald 1992), all experiments were performed in the presence of 40 \( \mu \)M amino-5-phosphonovaleric acid (AP-5). We observed that 10 \( \mu \)M PhTx did not affect field excitatory postsynaptic potential (fEPSP) amplitude (Fig. 1, A and C; \( n = 4 \) slices). In some experiments, PhTx promoted the appearance of population spikes (Fig. 1C), presumably because it blocked disinaptic inhibition (Toth and McBain 1998), indicating that the PhTx was pharmacologically active within the slice. Likewise, NASPM (200 \( \mu \)M) had a very small effect on fEPSP slope (79 ± 1% of control after 20 min, \( n = 16 \) slices; Fig. 1A),
consistent with the results of Noh et al. (2005). Some types of Ca\textsuperscript{2+}-permeable AMPARs are insensitive to PhTx (Osswald et al. 2007), however, and recent evidence suggests that endogenous polyamines block Ca\textsuperscript{2+}-permeable AMPARs in CA1 pyramidal neurons and therefore occlude the effects of PhTx (Rozov et al. 2012). Indeed, several reports suggest that the endogenous polyamine, spermine, interacts with Ca\textsuperscript{2+}-permeable AMPARs and can inhibit hippocampal excitatory synaptic transmission when applied either intra- or extracellularly (Discaena et al. 1994; Ferchmin et al. 1995; Rozov et al. 2012). Because Ca\textsuperscript{2+}-permeable AMPARs might be selectively enriched in s. radiatum (Toomim and Millington 1998), we performed these experiments using a two-pathway design in which one extracellular stimulating and recording electrode pair was placed in s. radiatum and another independent pair was placed in s. oriens so that AMPAR-mediated fEPSPs could be assessed at apical and basal synapses alternatingly in the same slices. Bath application of 100 μM spermine reduced the slope of s. radiatum fEPSPs by ~50% without affecting s. oriens fEPSPs (Fig. 1B; n = 6 slices), whereas application of 25 μM spermine reduced fEPSP slope in s. radiatum to 76 ± 2% of control (Fig. 1D; n = 11 slices). Extracellular application of spermine did not reduce fEPSPs in the absence of stimulation, and only became effective when stimulation was restarted (Fig. 1B; n = 3 slices), as is consistent with the use-dependent polyamine block of recombinant GluA2-lacking AMPARs (Washburn and Dingledine 1996).

We also evoked EPSCs in rat hippocampal slice cultures with synaptic stimulation (0.05–0.1 Hz) delivered alternatingly in s. radiatum and s. oriens using two stimulation pipettes placed within 5–10 μm of the Alexa Fluor 568-filled dendrites of voltage-clamped CA1 cells. Extracellular application of spermine (25 μM) reversibly depressed the amplitude of synaptic EPSCs evoked with stimulation in s. radiatum (mean = 72 ± 8% of control, n = 6 cultures; \( P < 0.01 \), Mann-Whitney \( U \) test) but did not significantly decrease synaptic EPSCs elicited with stimulation in s. oriens (Fig. 2; n = 5 cultures).

These results could be explained by a selective pre- or postsynaptic action of spermine. Specifically, apical dendritic synapses might contain some Ca\textsuperscript{2+}-permeable AMPARs that are sensitive to spermine but not PhTx or NASPM. Alternatively, spermine, but not PhTx or NASPM, might inhibit some presynaptic channels and thereby reduce transmitter release probability specifically at Schaffer collateral synapses in s. radiatum. As an independent test for the presence of Ca\textsuperscript{2+}-permeable AMPARs in the apical dendrites of CA1 pyramidal cells in adult rat brain, we next used the Co\textsuperscript{2+}-loading approach, as described by Auousseau et al. (2012). Acute hippocampal slices were placed in saline containing 5 mM CoCl\textsubscript{2} and 10 mM glutamate for 20 s to activate AMPARs followed by 40 s in saline containing Co\textsuperscript{2+} alone. This 20-s-on/40-s-off cycle was repeated 10 times over 10 min. Both solutions contained CTZ (30 μM) to minimize AMPAR desensitization. Slices were then fixed, resectioned, and stained for the presence of Ca\textsuperscript{2+} using silver intensification. Consistent with the electrophysiological results, strong loading of Co\textsuperscript{2+} was observed in dendrites in s. radiatum, but not s. oriens (Fig. 3; n = 6 slices), as well as in presumed interneurons located outside of s. pyramidalae. Glutamate-induced Co\textsuperscript{2+} loading was negligible when performed in the presence of 25 μM spermine (Fig. 3; n = 6 slices) and was greatly reduced in the absence of CTZ.

These data support the localization of Ca\textsuperscript{2+}-permeable, polyamine-sensitive AMPARs (or kainate receptors) potentially in the apical dendrites of CA1 pyramidal cells.

**Differences in AMPAR-mediated responses from apical and basal spines.** We further tested the hypothesis that some AMPARs in single dendritic spine heads are Ca\textsuperscript{2+}-permeable using microphotolysis of caged glutamate in the presence of TTX with NMDARs and GABA\textsubscript{A} receptors blocked pharmacologically (Bagal et al. 2005). This method has the advantage of activating postsynaptic AMPARs without involving presynaptic factors. The UV light was focused to a nearly diffraction-limited spot (~1-μm diameter) and likely activated both synaptic and extrasynaptic receptors confined to the targeted spine head. We focused the laser spot at dendritic spines located on apical dendrites, extending into s. radiatum, or basal dendrites, extending into s. oriens. phEPSCs recorded from either apical or basal dendritic spines were eliminated by 6,7-dinitroquinoxaline-2,3-dione (DNQX; 40 μM; data not shown), indicating that they were mediated solely by AMPARs. There were no significant differences in the mean amplitudes or rates of rise of phEPSCs elicited from spines on dendrites in apical or basal dendrites (unpaired \( t \)-test; n = 21, 27 spines; Fig. 4, A and B). The decay of AMPAR-mediated phEPSCs at both locations was well-fit with a single exponential. The mean decay time constant of phEPSCs elicited from basal spines was faster than for phEPSCs from apical spines (\( P < 0.01 \), unpaired \( t \)-test). Bath application of CTZ (10 μM) to slow AMPAR desensitization had no effect on amplitudes or rates of rise of phEPSCs elicited from either apical or basal dendritic spines but roughly doubled their decay time constants (\( P < 0.05 \), paired \( t \)-test; n = 5, 3; Fig. 4C). The decay of basal spine phEPSCs remained faster than that of apical spine phEPSCs in the presence of CTZ.

There was a strong correlation between the volume of the dendritic spine head targeted with the laser and the amplitude of phEPSCs elicited from spines on dendrites in apical or basal dendrites (Fig. 5, A and B).
of the resulting phEPSC (Fig. 4D) at both apical and basal spines, although the slope was slightly steeper for basal spines. AMPAR “silent” synapses, which express NMDARs but lack functional AMPARs, have been observed in acute and cultured hippocampal slices (Isaac et al. 1995; Liao et al. 1995; Montgomery et al. 2001). However, after photostimulation of many hundreds of dendritic spines in cultured slices for 14 days in vitro, we have never observed a single instance in which a mature spine, having a clearly distinguishable head and neck region, failed to respond to photorelease of glutamate with an AMPAR-mediated phEPSC, provided the health of the cell could be established by eliciting phEPSCs from other spines.

**Apical spine phEPSCs display properties of Ca^{2+}-permeable AMPARs.** Most Ca^{2+}-permeable, GluA2-lacking AMPARs display a polyamine-mediated, rectifying I–V, hence lack of rectification is commonly taken as evidence that these receptors are absent (Adesnik and Nicoll 2007; Plant et al. 2006; but see: Bowie 2012; Rozov et al. 2012). We therefore compared the rectification of phEPSPCs elicited from apical and basal spines using pipette solutions that contained 10 μM spermine. Only dendritic spines within 50 μm of the somatic recording electrode were sampled, and cesium was used in the pipette solution to minimize voltage- and space-clamp errors. The rectification index (RI) was measured as the phEPSC amplitude at +40 mV divided by its amplitude at −80 mV so that, assuming a reversal potential of 0 mV, a linear I–V would have an RI of 0.5. No significant difference in the RIs of phEPSCs from apical spines and basal spines was detected, although stronger rectification was observed at apical spines (apical RI = 0.37 ± 0.03, n = 15, vs. 0.44 ± 0.03, n = 8; P < 0.05, Mann-Whitney U test; Fig. 5). There was no difference in the rates of rise or decay time constants of phEPSCs at 80 and +40 mV. The observation of RIs <0.5 might suggest that apical and basal spines express some Ca^{2+}-permeable AMPARs. Alternatively, the currents often failed to reverse at 0 mV (mean current at 0 mV = 3.9 ± 1.0 and 1.7 ± 0.3 pA for apical and basal spines, n = 7 and 3, respectively), suggesting that these RI measurements might be compromised by imperfect voltage control at the stimulated spine. We therefore tested several other predictions of the presence of Ca^{2+}-permeable AMPARs.

Ca^{2+}-permeable, polyamine-sensitive AMPARs display PPF due to activity-dependent relief from intracellular polyamine block (Rozov et al. 1998; Rozov and Burnashev 1999;
then the PPR should be increased at depolarized membrane potentials because of the increased block of Ca\(^{2+}\)-permeable AMPARs by intracellular polyamines at those potentials. We tested this prediction by depolarizing the membrane from −80 to +40 mV in cells recorded with spermine-containing intracellular solutions and found that this significantly increased the PPR, measured at an ISI of 20 ms (PPR = 1.64 ± 0.04 at −80 vs. 2.03 ± 0.09 at +40 mV; n = 5; P < 0.01; Fig. 7A). Even with imperfect voltage-clamp, the voltage dependence of the PPR at apical spines suggests that relief of polyamine block accounts for the observed PPF.

Differences in PPR at apical and basal spines might reflect differences in the relative proportion of polyamine-sensitive AMPARs or differences in polyamine-independent processes, such as receptor mobility (Heine et al. 2008) or receptor desensitization. The PPR of phEPSCs at basal spines was not affected significantly by application of CTZ (PPR = 1.2 ± 0.1 before and 1.3 ± 0.2 after CTZ; n = 4; paired t-test). The difference in facilitation of phEPSCs at the two locations cannot therefore be attributed to differences in AMPAR desensitization. If PPF is larger at apical spines than basal spines because apical spines have more Ca\(^{2+}\)-permeable AMPARs than basal spines, then the PPR of basal spines should be less voltage-dependent than apical spines. Indeed, the PPR, measured at an ISI of 20 ms, at basal spines was not significantly different at the two membrane potentials (PPR = 1.23 ± 0.08 at −80 mV vs. 1.18 ± 0.08 at +40 mV; n = 8; not significant, paired t-test; Fig. 7A). The smaller voltage dependence of PPF at basal spines is consistent with a smaller contribution of Ca\(^{2+}\)-permeable AMPARs.

We next tested the effects of extracellularly applied polyamines. If apical spines contain Ca\(^{2+}\)-permeable AMPARs, then apical dendritic spine phEPSCs should be depressed by extracellular polyamines (Washburn and Dingledine 1996). Bath application of spermine (50 μM) produced a 70% decrease in the amplitude of apical spine phEPSCs (mean amplitude = 24.1 ± 6.6 pA before vs. 6.9 ± 1.6 pA after spermine; n = 8; P < 0.05, paired t-test; Fig. 7B).

If the reduction in apical spine phEPSC amplitude by spermine is caused by block of Ca\(^{2+}\)-permeable AMPARs, then the phEPSC PPR should increase because postsynaptic PPF results from the relief of both intra- and extracellular polyamine block (Rozov and Burnashev 1999). Indeed, the decrease in phEPSC amplitude was accompanied by a significant increase in PPR at apical spines (PPR = 1.56 ± 0.09 before vs. 2.11 ± 0.15 after spermine; n = 8; P < 0.005, paired t-test). In contrast, extracellular spermine did not alter the amplitude or PPR of phEPSCs elicited from basal spines significantly (phEPSC amplitude = 95 ± 2% of control; PPR = 108 ± 5% of control; n = 6; paired t-test; Fig. 7B).

Some apical AMPARs are resistant to block of GluA2 insertion. Synaptic delivery of GluA2-containing, but not GluA2-lacking, AMPARs is mediated through a cytoplasmic interaction with N-ethylmaleimide-sensitive factor (NSF; Lüthi et al. 1999; Nishimune et al. 1998). When applied intracellularly, a peptide consisting of the NSF-binding domain of GluA2, pep2m, competes with the endogenous GluA2 ligand for NSF and disrupts replenishment of GluA2-containing receptors, thereby depleting the synapses of putative GluA2-containing receptors. We observed that dialysis of pep2m (150 μM) into the cell from the patch pipette decreased AMPAR-mediated currents at both apical (Fig. 8A) and basal spines within 15 min after break-in to the whole cell recording mode. In agreement with previous studies in acute hippocampal slices (Lüthi et al. 1999; Nishimune et al. 1998), the decline in apical

Fig. 4. Comparison of the basic properties of photolysis-induced EPSCs (phEPSCs) at single dendritic spines on apical and basal dendrites. A: averaged phEPSCs at single apical and dendritic spines. B: mean amplitude, rate of rise, and decay time constant of phEPSCs at apical and basal spines (n = 21, 27 spines). Only the difference in decay time constant was significant (P < 0.01, unpaired t-tests). C: cyclothiazide (CTZ) prolongs apical and basal spine responses to similar extents. Responses of a basal spine to photostimulation before and after application of CTZ are shown above. Summary data below illustrate that CTZ decreased the decay rate of phEPSCs at both basal and basal spines significantly (*P < 0.05, paired t-test; n = 5, 3). D: the amplitude of responses at individual apical (black) and basal (gray) spines (n = 13, 15 spines, respectively) is plotted as a function of spine head volume. In both cases, the relationship was linear (r = 0.64 and 0.69, respectively). In this and all subsequent figures, arrowhead indicates delivery of UV pulse.

Shin et al. 2005). We therefore tested the effects of paired photostimuli in recordings with pipette solutions containing 10 μM spermine. Responses were collected using a two-stimulus protocol and an ISI that varied between 10 and 200 ms (Fig. 6).

The PPR was then calculated by taking the mean amplitude of the second responses and dividing them by the mean amplitude of the first responses. The amplitude of the second response was consistently greater than the amplitude of the first response at ISIs of 10–20 ms at apical dendritic spines. Basal spines also displayed PPF but significantly less than in apical spines (for ISI = 10 ms, PPR = 1.72 ± 0.06 at apical spines vs. 1.34 ± 0.05 at basal spines; n = 21, 27; P < 0.001, Mann-Whitney U test).

If the PPF of apical spine responses results from a relief of endogenous polyamine block of Ca\(^{2+}\)-permeable AMPARs, then the PPR should be increased at depolarized membrane potentials because of the increased block of Ca\(^{2+}\)-permeable AMPARs by intracellular polyamines at those potentials. We tested this prediction by depolarizing the membrane from −80
spine phEPSC amplitude reached a steady-state of ~50% of the control amplitude (mean amplitude at 16 min = 45 ± 3% of starting amplitude; n = 5 cells; Fig. 8B). In striking contrast, however, pep2m almost eliminated phEPSCs elicited from basal spines within 15 min (mean amplitude at 16 min = 10 ± 2% of starting amplitude; n = 5 cells; Fig. 8B). The decrease at basal spines was significantly greater than the decrease at apical spines (P < 0.05, Mann-Whitney U test). Dialysis of the scrambled control peptide, pep4c (150 μM), from the pipette had no effect on the amplitude of phEPSCs elicited from apical spines (mean amplitude 16 min after = 98 ± 2% of starting amplitude; n = 3) or basal spines (mean amplitude 16 min after = 98 ± 3% of starting amplitude; n = 5). We conclude that a larger proportion of AMPARs at basal spines depend on GluA2-dependent recycling than at apical spines.

There are two possible explanations for the partial reduction in apical spine phEPSC amplitude caused by pep2m. The remaining phEPSC could be mediated by GluA2-containing AMPARs that are not retrieved after block of GluA2 insertion (Lüthi et al. 1999; Nishimune et al. 1998). If this hypothesis is true, then there should be no change in PPR as the phEPSC amplitude declines. Alternatively, the persistent phEPSC could be mediated largely by AMPARs that are insensitive to pep2m because they lack GluA2 subunits. If this hypothesis is true, then the PPR of these phEPSCs should increase as removal of GluA2-containing AMPARs increases the fraction of the current mediated by GluA2-lacking AMPARs. Indeed, we observed an increase in the PPR of apical spine phEPSCs to 151 ± 23% of the control value (n = 5; P < 0.05, paired t-test) as the dialysis with pep2m caused them to become depressed (Fig. 8, A and C). At basal spines, in contrast, there was no significant change in the PPR of phEPSCs (PPR = 101 ± 7% of control, n = 5). These results are thus consistent with the hypothesis that apical spines express a considerable fraction of GluA2-lacking AMPARs (presumably GluA1 heteromers). The 50% reduction in apical spine phEPSCs resulting from pep2m dialysis suggests that perhaps as much as half of the current generated at apical spines is mediated by GluA2-lacking AMPARs.

Ca\(^{2+}\) influx through AMPARs at apical spines. If Ca\(^{2+}\)-permeable, polyamine-sensitive GluA2-lacking AMPARs are present at apical dendritic spines, then AMPAR activation should be accompanied by a local, polyamine-sensitive increase in the intracellular Ca\(^{2+}\) concentration. We anticipated that these responses would be small and difficult to detect because of the low Ca\(^{2+}\)-to-Na\(^+\) permeability ratio of the channels, their short open time, and the small number of channels likely to be present at a single spine. Whole cell recordings from CA1 cells were made with pipette solutions containing the high-affinity Ca\(^{2+}\) indicator OGB-1 (40–80 μM; Fig. 9). As a positive control for the health of the cell and the sensitivity of the Ca\(^{2+}\) imaging conditions, phEPSCs were first elicited with the cell voltage-clamped at −30 mV to relieve Mg\(^{2+}\)-block of NMDARs. Only spines displaying clear responses under these conditions were studied further. To isolate potential AMPAR-mediated Ca\(^{2+}\) influx, antagonists of NMDARs (160 μM AP-5 and 40 μM MK-801; Fig. 9A) were applied together with a cocktail of antagonists to block Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR; 20 μM ryanodine and 1 μM thapsigargin), metabotropic GluRs (100 μM LY 341495), and R-, P/Q- and N-, T-, and L-type voltage-dependent Ca\(^{2+}\) channels (VDCCs; 0.3 μM SNX 482, 1 μM ω-conotoxin MVIIIC, 1 μM mibefradil, and 40 μM nimodipine, respectively).
Responses at \(-30\) mV were greatly reduced under these conditions. To increase the chances of detecting AMPAR-mediated Ca\(^{2+}\) influx, pairs of laser pulses were delivered at an ISI of 20 ms to relieve polyamine block and maximally activate Ca\(^{2+}\)-permeable AMPARs, as described above.

Under these conditions, significant increases (\(>5\%\) lasting \(>100\) ms) in OGB-1 emission were detected in 37 of 56 (66\%) of the apical spines examined (Fig. 9A). The mean change in OGB-1 fluorescence was 15 \(\pm\) 3\% (\(\Delta F/F; n = 10\) spines), considerably less than the 60\% increase in fluorescence elicited with single-spine NMDAR activation at \(-30\) mV. In addition, the duration of the intracellular Ca\(^{2+}\) elevation in the presence of the blocker cocktail was significantly shorter than for NMDAR-mediated Ca\(^{2+}\) responses (304 \(\pm\) 17 ms for AMPAR influx vs. 1,337 \(\pm\) 152 ms for NMDAR influx; \(n = 5, 5\); paired \(t\)-test, \(P < 0.005\)). Increases in OGB-1 emission (\(>5\%\) lasting \(>100\) ms) were detected in only 40\% of basal spines tested. When responses were obtained in both apical and basal spines, consistent with the hypothesis that the apical spine phEPSCs that persist after pep2m dialysis are mediated primarily by GluA2-lacking AMPARs, there was a significant increase in the PPR of apical spine phEPSCs (\(*P < 0.05,\) paired \(t\)-test), but not basal spine phEPSCs, after 15 min of dialysis.
apical and basal spines of the same cell, the integrals of the Ca$^{2+}$ signals were significantly larger in apical spines than in basal spines ($n = 5$; Fig. 9B).

Although the experiments were performed under voltage-clamp in the presence of antagonists of VDCCs, we tested the hypothesis that residual AMPAR-mediated depolarization activated some population of unblocked VDCCs (Bloodgood et al. 2009; Heine et al. 2008) by comparing responses at −75 and −55 mV. If VDCCs contribute, then Ca$^{2+}$ responses should be larger at −55 mV because it is closer to their activation threshold. We observed, however, that the integrals of the Ca$^{2+}$ responses were significantly greater at −75 than at −55 mV (Fig. 9B; $n = 4$; $P < 0.05$, paired $t$-test). This is consistent with Ca$^{2+}$-permeable AMPARs as the source of the Ca$^{2+}$ influx because the driving force for Ca$^{2+}$ influx via this pathway is greater at −75 mV.

Several other lines of evidence indicated that the responses of apical dendritic spines were mediated by Ca$^{2+}$ influx directly via Ca$^{2+}$-permeable AMPARs. First, Ca$^{2+}$ signals at apical dendritic spines were abolished by the competitive AMPAR antagonist DNQX (20 μM; $n = 4$), indicating that they were totally dependent on AMPAR activation (Fig. 9C). Second, the size of the Ca$^{2+}$ response integral was significantly larger when AMPAR channel open time was prolonged by CTZ (20–100 μM; $n = 7$ spines, 5 cells; $P < 0.05$, paired $t$-test).

Finally, if Ca$^{2+}$ responses in apical spines are mediated by Ca$^{2+}$-permeable AMPARs, then they should be blocked by...
extracellular application of spermine in a use-dependent manner. After recording baseline responses for 10 or more paired-pulse stimuli in control saline, spermine (50–100 μM) decreased the amplitude of the Ca\(^{2+}\) responses gradually (\(n = 6\) spines; Fig. 9C). As expected for a use-dependent block of AMPAR-gated channels, abolition of Ca\(^{2+}\) responses occurred more effectively when experiments were repeated in the presence of CTZ, which prevented the desensitization of AMPARs and therefore held channels open longer (\(n = 5\) spines). Ca\(^{2+}\) responses in control saline were stable with and without CTZ (\(n = 7\) spines each, pooled).

DISCUSSION

We conclude that apical dendritic spines in CA1 pyramidal cells possess a population of Ca\(^{2+}\)-permeable, polyamine-sensitive AMPARs in both acutely prepared and cultured hippocampal slices. Classically, AMPARs with these properties are found to lack GluA2 subunits, and we therefore suggest that GluA2 subunits are absent in a significant fraction of synaptic AMPARs on apical dendritic spines, rendering them Ca\(^{2+}\)-permeable and sensitive to voltage- and use-dependent block by polyamines. Nevertheless, we do not know the actual subunit composition of the receptors mediating our responses and cannot therefore exclude the possibility that these responses are mediated by GluA2-containing, polyamine-sensitive, Ca\(^{2+}\)-permeable AMPARs (Bowie 2012).

AMPAR-mediated Ca\(^{2+}\) influx. The sine qua non of GluA2-lacking AMPARs is Ca\(^{2+}\) permeability. We observed that many apical spines exhibited a Ca\(^{2+}\) signal in response to uncaging stimuli in the presence of a cocktail of NMDAR, mGluR, and VDCC antagonists as well as inhibitors of CICR. Several lines of experimental evidence confirm that these Ca\(^{2+}\) signals were mediated by AMPARs. First, they were abolished by DNQX. Second, they were smaller at –55 than at –75 mV. This is inconsistent with alternative explanations for the Ca\(^{2+}\) influx, such as an AMPAR-activated stimulation of VDCCs (Bloodgood et al. 2009; Heine et al. 2008). The larger signal at hyperpolarized potentials is consistent with greater Ca\(^{2+}\) influx through open GluA2-lacking AMPARs as the result of an increased driving force. Third, inhibiting AMPAR desensitization with CTZ increased the size of the Ca\(^{2+}\) signals. Finally, Ca\(^{2+}\) responses were inhibited by spermine in a use- and CTZ-sensitive manner. We observed that Ca\(^{2+}\) responses were reduced progressively after spermine was added, requiring more than eight stimuli for full block. When channel open time was increased by CTZ, the rate of inhibition by spermine was faster, requiring only six stimuli for full block. These results, taken together, support strongly the hypothesis that Ca\(^{2+}\)-permeable AMPARs are present at spines on apical dendrites.

Ca\(^{2+}\) signals were evident in 66% of apical spines. This could indicate that not all spines express Ca\(^{2+}\)-permeable AMPARs. Alternatively, the AMPAR-triggered Ca\(^{2+}\) signals we observed were small, at least fivefold less than the NMDAR-mediated signals at the same spines, and may have been below our detection level at some spines. Ca\(^{2+}\) signals mediated by AMPARs are expected to be smaller than NMDAR-mediated responses, due to the more rapid inactivation, shorter channel open times, and lower Ca\(^{2+}\)-to-Na\(^{+}\) permeability ratio of AMPARs. Ca\(^{2+}\) signals were less commonly observed at basal spines, and when they were, the integrals of the Ca\(^{2+}\) signals were significantly smaller than those at apical spines. These responses may be due to incomplete block of other sources of Ca\(^{2+}\) entry or to the presence of some Ca\(^{2+}\)-permeable AMPARs. Taken together with the lack of Co\(^{2+}\) loading in s. radiatum, measurements of Ca\(^{2+}\) signals support our conclusion that Ca\(^{2+}\)-permeable AMPARs are preferentially localized to apical spines.

Polypeptide block of AMPARs. We confirmed earlier observations that the polypeptides PhTx and NASPM have little or no effect on Schaffer collateral fEPSPs in s. radiatum in acutely prepared hippocampal slices from adult rats (Adesnik and Nicoll 2007; Plant et al. 2006; Rozov et al. 2012). Another polypeptide, spermine, did reduce Schaffer collateral fEPSPs, however, indicating that some of the receptors are polyamine-sensitive. Moreover, the spermine-induced inhibition of fEPSPs was use-dependent, as predicted for responses mediated by GluA2-lacking AMPARs. Spermine is an endogenous polypeptide that affects several ion channels, but our use of NMDAR antagonists and the range of spermine concentrations we used are more consistent with the high affinity of polyanimes for AMPARs (Washburn and Dingledine 1996) than with the low affinity of Ca\(^{2+}\) or K\(^{+}\) channels (Lopatin et al. 1994). Spermine also blocked glutamate-induced Co\(^{2+}\) loading in acute hippocampal slices, an indicator of Ca\(^{2+}\)-permeable AMPARs.

Inhibition of fEPSPs by spermine, but not PhTx, was unexpected because PhTx is a potent antagonist of Ca\(^{2+}\)-permeable AMPARs at many synapses (Washburn and Dingledine 1996), including both excitatory synapses on GABAergic interneurons (Toth and McBain 1998) and excitatory synapses in GluA2 knockout mice (Adesnik and Nicoll 2007; Rozov et al. 2012). However, the PhTx molecule is much larger than spermine (7.5 vs. 5 Å) and has a slower binding rate than spermine (Bowie et al. 1998). Thus PhTx may not have equal access to all Ca\(^{2+}\)-permeable channels. The sensitivity of Schaffer collateral CA1 synapses to PhTx has only been observed when endogenous polyanimes were dialyzed from CA1 pyramidal cells and not before dialysis or when spermine was contained in the patch solution (Rozov et al. 2012). There is, however, evidence for PhTx-insensitive, Ca\(^{2+}\)-permeable AMPARs in the developing rat retina (Osswald et al. 2007). This evidence, combined with our other data, argues that PhTx insensitivity does not provide unambiguous confirmation of a lack of Ca\(^{2+}\)-permeable AMPARs at a given synapse.

Focal photolysis of caged glutamate at single dendritic spines made possible critical tests of the hypothesis that some AMPARs on CA1 pyramidal neurons are Ca\(^{2+}\)-permeable. Because excitatory synapses on basal dendrites in acute and cultured slices were insensitive to spermine, we hypothesized that these synapses expressed fewer Ca\(^{2+}\)-permeable AMPARs and could thus serve as controls for tests on apical spines. Kinetic analysis revealed that basal spine phEPSCs decayed more quickly than apical phEPSCs. Deactivation and desensitization are faster for GluA2-lacking AMPARs (Geiger et al. 1995). If there is a greater number of Ca\(^{2+}\)-permeable AMPARs at apical spines, as we did not OSM among experiments indicate, then it might be expected that apical spine phEPSCs would decay faster than basal spine phEPSCs. This was not observed.

Rectification of GluA2-lacking AMPARs is attributed to voltage-dependent channel block by intracellular polyanimes. Several studies of AMPAR-mediated currents in CA1 neurons
failed to detect inward rectification in the absence of added intracellular polyamines (Adesnik and Nicoll 2007; Hestrin et al. 1990; Plant et al. 2006; Rozov et al. 2012), suggesting the absence of Ca\(^{2+}\)-permeable AMPARs. However, when endogenous polyamines were dialyzed into hippocampal CA1 neurons from wild-type mouse hippocampi, inward rectification was observed, and this I–V deviated from the nonrectifying I–V observed in neurons from mutant mice lacking GluA1 or expressing GluA1 with a point mutation in the Q/R site (Rozov et al. 2012). It should be noted that all amacrine cells in the retina express Ca\(^{2+}\)-permeable AMPARs that are insensitive to extracellular PhTx and do not rectify (Osswald et al. 2007), demonstrating that Ca\(^{2+}\) permeability is not always synonymous with polyamine-sensitivity (for review, see Bowie 2012). We used single-spine photolysis to assess the rectification properties of AMPARs and observed modest rectification of pEPSCs at both apical and basal spines, like those reported for synaptic responses in some studies (Hayashi et al. 2000). We noted that the pEPSC reversal potential was more negative than 0 mV, suggesting inadequate control of spine membrane potential at depolarized voltages. These experiments were thus not conclusive. Our finding of strong voltage- and spermine-sensitive PPF indicates that Ca\(^{2+}\)-permeable AMPARs in CA1 cell apical spines are sensitive to intra- and extracellular polyamines. To our knowledge, this profile of characteristics is uniquely associated with GluA2-lacking AMPARs, but positive identification must await molecular analysis of these synapses.

Pep2m, a peptide derived from the COOH-terminal region of GluA2 that interacts with the trafficking protein NSF, prevents the constitutive cycling of GluA2-containing, but not GluA2-lacking, AMPARs to the synapse (Lüthi et al. 1999; Nishimune et al. 1998). Pep2m abolished AMPAR-mediated pEPSCs elicited from basal spines when dialyzed into CA1 pyramidal cells but depressed pEPSC amplitudes from apical spines by only ~50%. The decrease in pEPSC amplitude was accompanied by an increase in PPF. These data thus lend further support to the hypothesis that the Ca\(^{2+}\)-permeable, polyamine-sensitive responses that we have recorded are mediated by GluA2-lacking AMPARs and that as much as half of the total AMPAR-mediated current may be carried by these AMPARs. If these channels are GluA1 homomers, then their number may be small because of their relatively large single-channel conductance (Rozov et al. 2012; Swanson et al. 1997).

Intracellular polyamines block closed GluA2-lacking AMPARs and are released when the channels open (Bowie and Mayer 1995; Koh et al. 1995), giving rise to PPF (Rozov et al. 1998; Rozov and Burnashev 1999). We observed that the PPRs of pEPSCs recorded from apical spines were considerably larger than those from basal spines. In addition, the PPF observed at apical spines was increased at depolarized potentials, whereas the PPF at basal spines was voltage-independent. Furthermore, extracellular spermine reduced the amplitude of pEPSCs recorded at apical spines and increased the PPF of these pEPSCs, whereas the amplitude and PPR of pEPSCs recorded from basal spines were not affected. These data further support the hypothesis that apical spines contain Ca\(^{2+}\)-permeable, polyamine-sensitive AMPARs. Although basal synapses were polyamine-insensitive, we observed some evidence of Ca\(^{2+}\) permeability, the source of which is unknown. It is interesting to speculate that some basal synapses may express Ca\(^{2+}\)-permeable, GluA2-containing AMPARs (Bowie 2012).

Comparison with previous studies. Our conclusion that Ca\(^{2+}\)-permeable AMPARs are present on apical dendritic spines of CA1 pyramidal neurons is the opposite of that reached in several other studies (Adesnik and Nicoll 2007; Gray et al. 2007; Plant et al. 2006). We have employed a combination of methods that has not been used to test this hypothesis previously, including single-spine microphotolysis of caged glutamate and microfluorometric Ca\(^{2+}\) imaging. In general, the previous studies based their conclusions on negative findings, such as a lack of rectification and a lack of PhTx sensitivity. In contrast, Rozov et al. (2012) demonstrated that when endogenous polyamines were dialyzed out from CA1 pyramidal neurons, AMPAR-mediated EPSCs increased in amplitude over time, became sensitive to PhTx, and exhibited decreased rectification. Thus endogenous polyamines may account for the apparently small contribution of Ca\(^{2+}\)-permeable AMPARs to the synaptic I–V, as concluded from the nonrectifying I–V curve described in previous studies (Rozov et al. 2012). Wenthold et al. (1996) demonstrated that GluA1/2 and GluA2/3 heteromers constitute the majority of hippocampal AMPARs but also noted that 10% consisted of homomeric GluA1. CA1 pyramidal cells are capable of synthesizing GluA1 homeric receptors and inserting them into the synaptic plasma membrane (Lu et al. 2009). Indeed, insertion of GluA2-lacking AMPARs is a major homeostatic response to reduced synaptic activity (Harms et al. 2005; Hou et al. 2008; Sutton et al. 2006; Thiagarajan et al. 2005).

He et al. (2009) have demonstrated that GluA2-lacking AMPARs are present at perisynaptic sites that were likely to be activated by glutamate microphotolysis in our experiments. Assuming that spermine inhibits EPSCs by acting solely at Ca\(^{2+}\)-permeable AMPARs, our evidence that spermine reduces fEPSPs in s. radiatum of adult rat hippocampal slices indicates that at least some spermine-sensitive Ca\(^{2+}\)-permeable AMPARs are accessible to synthetically released glutamate under our stimulation conditions. Further experiments, such as manipulations of intracellular polyamine concentrations, would be required to test this hypothesis more fully. Ca\(^{2+}\)-permeable AMPARs are regulated developmentally in the neocortex, decreasing in number with maturity (Shin et al. 2005). The organotypic hippocampal slice cultures used in our photolysis experiments may mature more slowly than the intact brain; however, we observed comparable sensitivity of s. radiatum synaptic responses to spermine in both cultured and acute hippocampal slices as well as significant glutamate-induced, spermine-sensitive Co\(^{2+}\) loading in acute slices.

Conclusion. The presence of Ca\(^{2+}\)-permeable AMPARs in the dendritic spines of apical dendrites is of considerable functional importance. The amount of Ca\(^{2+}\) that enters the cell via this route is low, however, as demonstrated in our Ca\(^{2+}\) imaging results, so the existence of GluA2-lacking AMPARs is consistent with the overwhelming evidence that NMDAR-mediated Ca\(^{2+}\) influx is the predominant source of synaptic Ca\(^{2+}\) for conventional synaptic plasticity. Nevertheless, Ca\(^{2+}\)-permeable AMPARs may take part in synaptic plasticity under other conditions, such as after chronic inactivity, and may be upregulated under pathological conditions (e.g., Liu and Zukin...


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