Impact of subthreshold membrane potential on synaptic responses at dendritic spines of layer 5 pyramidal neurons in the prefrontal cortex

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Seong HJ, Behnia R, Carter AG. Impact of subthreshold membrane potential on synaptic responses at dendritic spines of layer 5 pyramidal neurons in the prefrontal cortex. J Neurophysiol 111: 1960–1972, 2014. First published January 29, 2014; doi:10.1152/jn.00590.2013.—Glutamatergic inputs onto cortical pyramidal neurons are received and initially processed at dendritic spines. AMPA and NMDA receptors generate both synaptic potentials and calcium (Ca) signals in the spine head. These responses can in turn activate a variety of Ca, sodium (Na), and potassium (K) channels at spines. In principle, the roles of these receptors and channels can be strongly regulated by the subthreshold membrane potential. However, the impact of different receptors and channels has usually been studied at the level of dendrites. Much less is known about their influence at spines, where synaptic transmission and plasticity primarily occur. Here we examine single-spine responses in the basal dendrites of layer 5 pyramidal neurons in the mouse prefrontal cortex. Using two-photon microscopy and two-photon uncaging, we first show that synaptic potentials and Ca signals differ at resting and near-threshold potentials. We then determine how subthreshold depolarizations alter the contributions of AMPA and NMDA receptors to synaptic responses. We show that voltage-sensitive Ca channels enhance synaptic Ca signals but fail to engage small-conductance Ca-activated K (SK) channels, which require greater numbers of inputs. Finally, we establish how the subthreshold membrane potential controls the ability of voltage-sensitive Na channels and K channels to influence synaptic responses. Our findings reveal how subthreshold depolarizations promote electrical and biochemical signaling at dendritic spines by regualting the contributions of multiple glutamate receptors and ion channels.

prefrontal cortex; pyramidal neuron; dendrite; spine; synapse; calcium signaling; two-photon microscopy; two-photon uncaging

GLUTAMATERGIC INPUTS evoke excitatory postsynaptic potentials (EPSPs) and synaptic calcium (Ca) signals at dendritic spines of cortical pyramidal neurons. These synaptic responses are mediated by a complex assortment of glutamate receptors and ion channels (Bloodgood and Sabatini 2007a). In other neurons, the subthreshold membrane potential can strongly influence synaptic responses by changing the contributions of receptors and channels (Carter and Sabatini 2004). In the cortex this potential spans 20 mV from rest to near threshold (Steriade et al. 1993), but the functional impact on single-spine responses remains unknown. Understanding this importance is essential, as local EPSPs and synaptic Ca signals regulate dendritic excitability and synaptic plasticity (Brancos and Hausser 2010; Sjostrom et al. 2008).

Dendritic spines function as electrical and biochemical compartments, owing to the high resistance of the spine neck (Bloodgood and Sabatini 2005; Harnett et al. 2012; Svoboda et al. 1996). Consequently, activation of AMPA receptors (AMPA-Rs) evokes local EPSPs in the spine head that reach tens of millivolts (Palmer and Stuart 2009; Tsay and Yuste 2004). Moreover, activation of NMDA receptors (NMDA-Rs) generates synaptic Ca signals that are spatially restricted to spines (Koester and Sakmann 1998; Kovalchuk et al. 2000; Sabatini et al. 2002). In principle, subthreshold depolarizations can regulate these responses by decreasing the driving force of AMPA-Rs and increasing Ca influx through NMDA-Rs (Carter and Sabatini 2004). However, because local EPSPs evoked by AMPA-Rs also enhance synaptic Ca signals via NMDA-Rs (Bloodgood et al. 2009; Grunditz et al. 2008), it is challenging to predict the overall influence of the subthreshold membrane potential on single-spine responses.

Local EPSPs in the spine head have long been predicted to activate voltage-sensitive ion channels (Miller et al. 1985; Perkel and Perkel 1985; Segev and Rall 1988, 1998). However, the influence of these channels has primarily been examined with compound EPSPs evoked by multiple inputs. Voltage-sensitive sodium (Na) and Ca channels provide an additional depolarization that enhances EPSPs (Magee and Johnston 1995; Markram and Sakmann 1994; Miyakawa et al. 1992; Stuart and Sakmann 1995). In contrast, A-type potassium (K) channels create an opposing hyperpolarization that suppresses EPSPs (Cai et al. 2004; Losonczy and Magee 2006). Na, Ca, and K channels also bidirectionally regulate synaptic Ca signals, which are evoked in both spines and dendrites by multiple inputs. Importantly, the impact of these channels can be strongly influenced by subthreshold depolarization, which can recruit persistent Na channels (Lipowsky et al. 1996) and inactivate A-type K channels (Hoffman et al. 1997).

While a variety of receptors and channels can contribute to synaptic responses, their roles at individual spines have been difficult to determine. Two-photon uncaging mimics isolated inputs by circumventing presynaptic release and directly activating glutamate receptors on spines (Matsuzaki et al. 2001). This approach has revealed that Na channels also enhance EPSPs at spines (Araya et al. 2007), and this effect becomes greater at near-threshold potentials (Carter et al. 2012). Similarly, Ca channels directly contribute to both EPSPs and synaptic Ca signals at spines, as initially observed with electrical stimulation (Denk et al. 1995). Ca influx through these channels can also engage small-conductance Ca-activated K (SK) channels, which hyperpolarize the spine head and dampen synaptic responses (Bloodgood and Sabatini 2007b; Ngo-Anh et al. 2005). In principle, A-type K channels could similarly suppress local signaling, but their impact on synaptic
responses at spines remains unknown. One untested hypothesis is that A-type K channels are engaged at resting potentials, whereas activation of SK channels becomes more prominent when synaptic Ca signals are larger at near-threshold potentials.

Here we examine the impact of the subthreshold membrane potential on synaptic responses at spines in the basal dendrites of layer 5 pyramidal neurons in the mouse prefrontal cortex. Interactions between glutamate receptors and ion channels in these neurons are thought to enable persistent activity and shape cognitive function (Durstewitz et al. 2000; Wang 2001). We use a combination of two-photon microscopy and two-photon uncaging to study EPSPs and synaptic Ca signals at single spines. We first establish how subthreshold depolarizations alter the contributions of AMPA-Rs and NMDA-Rs to synaptic responses. We then determine that Ca channels enhance these responses whereas Ca-activated SK channels have a limited role. Finally, we show how the roles of both Na channels and A-type K channels depend on the subthreshold membrane potential. These findings illustrate how a dynamic interplay between multiple glutamate receptors and ion channels governs local signaling at dendritic spines.

MATERIALS AND METHODS

Slice preparation. Layer 5 pyramidal neurons were studied in acute slices of the prelimbic medial prefrontal cortex from male and female postnatal day 21–28 Swiss Webster mice as previously described (Chalifoux and Carter 2011b). All procedures followed protocols reviewed and approved by the New York University animal welfare committee. Mice were anesthetized with a lethal dose of ketamine and xylazine and perfused intracardially with ice-cold external solution containing (in mM) 65 sucrose, 76 NaCl, 25 NaHCO₃, 1.4 NaH₂PO₄, 25 glucose, 2.5 KCl, 1 CaCl₂, 5 MgCl₂, 0.4 Na-ascorbate, and 2 Na-pyruvate (295–305 mosM), bubbled with 95% O₂-5% CO₂. Coronal slices (300 μm thick) were cut in ice-cold external solution and transferred to artificial cerebral spinal fluid (ACSF) containing (in mM) 120 NaCl, 25 NaHCO₃, 1.4 NaH₂PO₄, 21 glucose, 2.5 KCl, 2 CaCl₂, 1 MgCl₂, 0.4 Na-ascorbate, and 2 Na-pyruvate (295–305 mosM), bubbled with 95% O₂-5% CO₂. Coronal slices recovered for 30 min at 24°C. All experiments were conducted at near-physiological temperatures (32–34°C). All solution chemicals were from Sigma.

Electrophysiology. Whole cell recordings were obtained from identified layer 5 pyramidal neurons located 450–550 μm from the pial surface. Borosilicate glass recording pipettes (2.5–4.5 MΩ) were filled with one of two internal solutions. Current-clamp recording internal solution contained (in mM) 135 K-glucuronate, 7 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 Mg2+ATP, and 0.4 Na-GTP (290–295 mosM, pH 7.3 with KOH). Voltage-clamp recording internal solution contained (in mM) 135 Cs-glucuronate, 10 HEPES, 10 Na-phosphocreatine, 4 Mg2+ATP, and 0.4 Na-GTP (290–295 mosM, pH 7.3 with CsOH). The internal solution also contained Alexa Fluor 594 (200 μM) to image morphology and fluo-4F (500 μM or 1 mM) or fluo-5F (200 μM or 500 μM) to detect Ca signals. For each experiment, Ca indicator concentration was chosen to ensure a high signal-to-noise ratio within the linear range (Yasuda et al. 2004). Series resistance was maintained at 12–25 MΩ, and cells were filled via the patch pipette for at least 15 min before imaging. All dyes were from Invitrogen.

Recordings were performed with a Multiclamp 700B amplifier. Physiologic data were sampled at 10 kHz and filtered at 4 kHz. Single action potentials (APs) were evoked by a 2-ms current pulse at 200 pA above the rheobase. Input resistance was monitored with a 500-ms current pulse of ~50 pA. For some experiments, a constant holding current was applied to maintain cells at subthreshold membrane potentials. Recordings were discarded if large changes in series resistance, input resistance, resting potential, or holding current were detected. Minimal stimulation of isolated synaptic inputs was performed with a small theta glass electrode with a 3- to 5-μm tip diameter placed <10 μm from the spine head (Chalifoux and Carter 2010). Stimulation of multiple synaptic inputs was performed with a larger theta glass electrode with a 7- to 15-μm tip diameter placed 15–20 μm from a dendritic segment (Chalifoux and Carter 2011b).

Two-photon microscopy. Intracellular Ca imaging and glutamate uncaging were achieved through a custom-made microscope as previously described (Carter and Sabatini 2004; Chalifoux and Carter 2010). For two-photon Ca imaging, line-scans were performed through dendrites and spines to monitor green (fluor-4FF or fluo-5F) and red (Alexa Fluor 594) fluorescence. Single line-scans were performed at 500 Hz. Multiple line-scans were performed at 100–250 Hz (2–5 spines) (Chalifoux and Carter 2011b). Raw Ca signals were measured as changes in green normalized to red (ΔG/R). These signals were later normalized off-line with saturated G/R (G/Rsat) to give units of ΔG/Gsat. Values for ΔG/Gsat were obtained with a sealed-tip thin-walled pipette filled with recording internal solution containing a saturating Ca concentration. ΔG/Gsat was measured at the end of each recording at the same temperature by placing the pipette directly above the soma position. A reference image was taken for each dendrite segment and interleaved between line-scans to correct for any drift in the x- and y-planes. Line-scans during current injection or uncaging were interleaved with line-scans without a stimulus to monitor photobleaching. Recordings were discarded if baseline Ca signals increased.

For minimal stimulation experiments, synaptic Ca signals were measured at activated spines and successes determined as responses greater than 3 standard deviations above the baseline noise. For two-photon uncaging experiments, 2.5 mM MN1-glutamate was applied to the bath and glutamate was photoreleased with a 1-ms pulse of 725-nm light delivered at 80 mW. Laser power was measured at the back focal plane of the objective and chosen to match the synaptic Ca signals evoked with minimal stimulation. Proximal mushroom spines were chosen within 120 μm of the soma, in order to maximize our ability to influence the local membrane potential, and within 30 μm of the surface of the slice, in order to minimize variability in local uncaging power. For each spine, the optimal uncaging position was determined as that which produced the largest test response in a hemicircle 0.5 μm away from the spine head.

Pharmacology. Synaptic experiments were performed in 10 μM d-serine to prevent NMDA-R desensitization. AP experiments were performed in 10 μM NBQX to block AMPA-Rs, 10 μM CPP to block NMDA-Rs, and 10 μM SR 95531 to block GABAA receptors. For most experiments, drugs were bath-applied. For some experiments, filtered drugs were diluted in ACSF, added to pipettes (1- to 3-μm tip diameter, placed 5–10 μm from the spine of interest), and applied at constant pressure (5–10 psi) with a picospritzer. The extent of puffing was confirmed in separate experiments using the green dye Alexa Fluor 488 (20 μM). The cocktail of Ca channel blockers was bath-applied and consisted of (in μM) 20 nimodipine, 10 mibefradil, 0.3 SNX-482, 1 w-conotoxin-MVIIC, 1 w-conotoxin-GVIA, and 0.2 w-agatoxin IVA (Chalifoux and Carter 2011a). Apamin was bath-applied at 100 nM. TTX was either bath-applied at 1 μM or puffed at 3 μM. 4-Aminopyridine (4-AP) was either bath-applied at 1 mM or puffed at 10 μM. Chemicals were from Tocris, Calbiochem, and Sigma.

Imaging and physiology data were acquired with National Instruments boards and custom software written in MATLAB (MathWorks). Offline analysis was performed with custom routines written in IGOR Pro (WaveMetrics). EPSP amplitudes were calculated as the 1-ms average around the peak of the baseline response. EPSP widths were calculated as the time interval
between the half-maximal amplitudes during the rising and falling phases. Ca signals were baseline subtracted and measured as the average amplitude of the first 250 ms after the stimulus. AP widths were calculated at 10% of the peak amplitude. In figures of average Ca signals, shutter artifact at the onset of uncaging pulse was blanked for display purposes. Data in the text and electrophysiology and imaging traces shown in figures are reported as means ± SE. Summary data in figures are shown as either bar graphs of mean ± SE or box plots of median, interquartile range, 10–90% range, and single data points. Differences between the median and the mean account for some discrepancies between values shown in figures and reported in the text. Significance was defined as \( P < 0.05 \) and determined with the nonparametric Wilcoxon signed-rank test, which makes no assumptions about the data distribution.

RESULTS

**Studying synaptic responses at dendritic spines.** We examined synaptic responses at spines in the basal dendrites of layer 5 pyramidal neurons in acute slices of mouse prefrontal cortex (Fig. 1A). We first used minimal electrical stimulation to establish the physiological range of synaptic Ca signals generated at spines (Chalifoux and Carter 2010; Mainen et al. 1999) (Fig. 1B). In whole cell recordings, we filled neurons with 1 mM fluo-4FF to measure synaptic Ca signals and 20 \( \mu \)M Alexa Fluor 594 to view their morphology. To maximize our ability to control the local membrane potential, we focused on spines within 120 \( \mu \)m of the soma. In voltage-clamp recordings at 0 mV, and in the presence of the AMPA-R blocker NBQX (10 \( \mu \)M), we stimulated isolated inputs with a small theta glass electrode and measured synaptic Ca signals at activated spines in successful trials (19.0 ± 2.2% \( \Delta G/G_{sat} \); \( n = 16 \) spines). In separate experiments, we then established the two-photon uncaging parameters needed to evoke equivalent Ca signals at single spines (Fig. 1C). Under the same recording conditions, we photoreleased bath-applied MNI-glutamate (2.5 mM) at spines with a 1-ms pulse of 725-nm light. We uncaged glutamate at 0.5 \( \mu \)m from the spine head, which evoked stable responses without photobleaching or photodamage. At each spine, we assessed a range of powers and determined the average power needed to evoke physiological Ca signals (80 \( \mu \)W = 20.4 ± 3.5% \( \Delta G/G_{sat} \); \( n = 12 \) spines) (Fig. 1D). For the remainder of our experiments, we then used these calibrated two-photon uncaging parameters to study synaptic responses at individual spines.

**Impact of subthreshold membrane potential.** Synaptic responses at single spines can be strongly influenced by the subthreshold membrane potential (Carter and Sabatini 2004), which in cortical pyramidal neurons ranges from −70 mV at rest to −50 mV near threshold (Steriade et al. 1993). Having established our two-photon uncaging parameters, we next assessed how synaptic responses vary across this potential range. Importantly, for these experiments, we used 500 \( \mu \)M fluo-5F to measure synaptic Ca signals in the linear range of the indicator (Yasuda et al. 2004). In voltage-clamp recordings, and in the presence of the NMDA-R blocker CPP (10 \( \mu \)M), we found that depolarization decreased AMPA-R uncaging-evoked excitatory postsynaptic currents (uEPSCs) (−70 mV = 33 ± 3 pA, −60 mV = 30 ± 2 pA, −50 mV = 27 ± 2 pA; \( n = 17 \) spines) (Fig. 2A), indicating a reduction in the driving force. In contrast, in the presence of NBQX (10 \( \mu \)M), depolarization increased NMDA-R synaptic Ca signals (−70 mV = 2.7 ± 0.5% \( \Delta G/G_{sat} \); −60 mV = 3.7 ± 0.6% \( \Delta G/G_{sat} \); −50 mV = 6.2 ± 1.1% \( \Delta G/G_{sat} \); \( n = 12 \) spines) (Fig. 2B), consistent with relief of magnesium (Mg) block.

We next assessed the impact of subthreshold membrane potential on synaptic responses in current-clamp recordings. In the absence of AMPA-R or NMDA-R blockers, we injected current to maintain neurons at −70 mV, −60 mV, and −50
membrane potential strongly influences synaptic potentials and Ca signals at individual spines, which could reflect changing contributions of a variety of voltage-sensitive receptors and channels.

**Multiple contributions of AMPA and NMDA receptors.** We next assessed the contributions of glutamate receptors to single-spine responses at resting and near-threshold potentials. In other pyramidal neurons, AMPA-Rs generate a local EPSP in the spine head (Bloodgood et al. 2009; Grunditz et al. 2008), whereas NMDA-Rs are primarily responsible for the synaptic Ca signal (Koester and Sakmann 1998; Kovalchuk et al. 2000; Sabatini et al. 2002). To assess the roles of these receptors, we bath-applied selective antagonists, which allowed us to make pairwise comparisons to baseline conditions. At resting potentials, we found that wash-in of NBQX (10 μM) eliminated uEPSPs (3 ± 2% of baseline, P = 0.004; n = 9 spines) and reduced synaptic Ca signals (58 ± 6% of baseline, P = 0.004) (Fig. 3A). In contrast, wash-in of CPP (10 μM) had little impact on uEPSP amplitude (88 ± 9% of baseline, P = 0.1; n = 9 spines) or uEPSP half-width (92 ± 15% of baseline, P = 0.7) but blocked most of the synaptic Ca signal (23 ± 4% of baseline, P = 0.004) (Fig. 3B). Blocking both AMPA-Rs and NMDA-Rs eliminated uEPSPs (−2 ± 2% of baseline, P = 0.03; n = 7 spines) and synaptic Ca signals (10 ± 3% of baseline, P = 0.03) (Fig. 3C). In control experiments, wash-in of ACSF confirmed stable recordings, with no change in uEPSP amplitude (94 ± 9% of baseline, P = 0.6; n = 13 spines), uEPSP half-width (126 ± 23% of baseline, P = 0.4), or synaptic Ca signals (99 ± 3% of baseline, P = 0.4) (data not shown). These findings indicate that glutamate receptors can have multiple influences on synaptic responses at individual spines. At resting potentials, AMPA-Rs are primarily responsible for uEPSPs but also enhance synaptic Ca signals. In contrast, NMDA-Rs are responsible for synaptic Ca signals but have a negligible electrogenic role.

Our voltage-clamp recordings suggest that the influences of AMPA-Rs may decrease and NMDA-Rs may increase closer to threshold. In neurons depolarized to near-threshold potentials, blocking AMPA-Rs continued to eliminate uEPSPs (3 ± 3% of baseline, P = 0.002; n = 10 spines) and reduce synaptic Ca signals (70 ± 7% of baseline, P = 0.004) (Fig. 4A). However, in contrast to our findings at resting potentials, we also observed a small residual EPSP with a slower time to peak. Blocking NMDA-Rs now reduced uEPSP amplitude (74 ± 6% of baseline, P = 0.01; n = 9 spines) and uEPSP

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**Fig. 2.** Single-spine responses depend on subthreshold membrane potential. A, top: voltage-clamp recordings of AMPA receptor (AMPA-R) uncaging-evoked excitatory postsynaptic currents (uEPSCs) evoked at different membrane potentials in the presence of CPP (10 μM). Thick lines indicate mean, and shaded regions indicate ±SE. Arrowhead indicates stimulus time. Bottom: summary of AMPA-R uEPSC amplitude as a function of holding potential (V_hold). B: similar to A for NMDA receptor (NMDA-R) Ca signals in the presence of NBQX (10 μM). C, top: current-clamp recordings of uncaging-evoked excitatory postsynaptic potentials (uEPSPs) evoked at different membrane potentials, in the absence of any glutamate receptor antagonists. Middle: summary of uEPSP amplitude as a function of membrane potential (V_m). Bottom: summary of uEPSP half-width as a function of membrane potential. D: similar to C for synaptic Ca signals. E: uEPSP half-width plotted against synaptic Ca signal amplitude at the different membrane potentials. Dashed line indicates fit to pooled data (r = 0.13). *Significance (P < 0.05).
Fig. 3. Glutamate receptor contributions at resting potentials. A: average uEPSPs (left) and synaptic Ca signals (center) evoked at resting potentials under baseline conditions (black) and after wash-in of NBQX (red). Thick lines indicate means, and shaded regions indicate ± SE. Arrowheads indicate stimulus time. Right: summary of impact of NBQX on uEPSP and synaptic Ca signal (uCa) amplitudes as % of baseline conditions shown as box plots of median (thick line), interquartile range (box), 10–90% range (whiskers), and single data points (open circles). B: similar to A for wash-in of CPP, also summarizing impact on uEPSP half-width. C: similar to A for wash-in of both NBQX and CPP. *Significance (P < 0.05).

half-width (62 ± 8% of baseline, P = 0.01) in addition to synaptic Ca signals (11 ± 2% of baseline, P = 0.004) (Fig. 4B). Blocking both AMPA-Rs and NMDA-Rs continued to eliminate uEPSPs (1 ± 3% of baseline, P = 0.008; n = 8 spines) and synaptic Ca signals (5 ± 2% of baseline, P = 0.008) (Fig. 4C). Finally, similar to resting potentials, wash-in of ACSF had no effect on uEPSP amplitude (95 ± 8% of baseline, P = 0.6; n = 9 spines), uEPSP half-width (103 ± 43% of baseline, P = 0.4), or synaptic Ca signals (94 ± 10% of baseline, P = 0.5) (data not shown). Together, these findings reveal how the roles of glutamate receptors at spines depend on the subthreshold membrane potential. At near-threshold potentials, AMPA-Rs shape both uEPSPs and synaptic Ca signals. In addition, NMDA-Rs assume an electrogenic role, influencing both the amplitude and time course of uEPSPs.

Ca channels enhance synaptic responses at spines. The ability of AMPA-Rs to enhance synaptic Ca signals suggested that local EPSPs within the spine head might also activate voltage-sensitive ion channels. Voltage-sensitive Ca channels are present throughout the dendrites and spines of pyramidal neurons and shape both electrical and biochemical signaling (Bloodgood and Sabatini 2007a). To assess the impact of these channels on synaptic responses, it was first useful to have an independent indicator of their presence. APs invade the dendrites of pyramidal neurons (Markram et al. 1995; Stuart and Sakmann 1994; Yuste and Denk 1995), where they evoke Ca signals in spines (Fig. 5A). We used 200 μM fluo-5F to measure AP Ca signals, which allowed us to record smaller transients in the linear range (Yasuda et al. 2004). To assess the impact of Ca channels, we bath-applied a cocktail of selective blockers (20 μM nimodipine, 10 μM mibebradil, 0.3 μM SNX-482, 1 μM ω-conotoxin-MVIIC, 1 μM ω-conotoxin-GVIA, and 0.2 μM ω-agatoxin IVA) (Chalifoux and Carter 2011a). In the presence of NBQX and CPP, we found that wash-in of this cocktail greatly reduced AP Ca signals (22 ± 1% of baseline, P < 0.0001; n = 36 spines), with no effect on the somatic AP waveform (amplitude = 103 ± 13% of baseline, P = 0.9; width = 114 ± 10% of baseline, P = 0.2; n = 7 cells) (Fig. 5B). These findings confirm that Ca channels contribute to AP Ca signals, suggesting that they could also influence synaptic responses at single spines.

In other pyramidal neurons, Ca channels can enhance synaptic responses generated by multiple inputs (Gillessen and Alzheimer 1997; Magee et al. 1995; Magee and Johnston 1995; Markram and Sakmann 1994; Miyakawa et al. 1992). These channels can also directly or indirectly contribute to responses at individual spines (Bloodgood and Sabatini 2007b; Denk et al. 1995; Egger et al. 2005; Grunditz et al. 2008; Reid et al.

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2001; Schiller et al. 1998). At resting potentials, we found that wash-in of the cocktail of Ca channel blockers had no impact on uEPSP amplitude (98 ± 6% of baseline, P = 0.7; n = 8 spines) or uEPSP half-width (107 ± 11% of baseline, P = 0.8) but significantly reduced synaptic Ca signals (78 ± 6% of baseline, P = 0.02) (Fig. 5C). Interestingly, this decrease in the synaptic Ca signal was similar in amplitude to the residual Ca signal after wash-in of CPP. Consistent with a role for Ca channels, in the presence of the cocktail of Ca channel blockers we found that wash-in of CPP (10 μM) now eliminated synaptic Ca signals (13 ± 4% of baseline, P = 0.002; n = 10 spines) but continued to have little impact on uEPSP amplitude (81 ± 8% of baseline, P = 0.6) or uEPSP half-width (95 ± 13% of baseline, P = 0.4) (data not shown). These findings indicate that Ca channels contribute to synaptic Ca signals but not uEPSPs at resting potentials.

The influence of Ca channels at spines can also strongly depend on the subthreshold membrane potential (Carter and Sabatini 2004). At near-threshold potentials, we found that wash-in of the cocktail of Ca channel blockers continued to reduce synaptic Ca signals (77 ± 4% of baseline, P = 0.0005; n = 12 spines) and now had a modest effect on uEPSPs (amplitude = 88 ± 4% of baseline, P = 0.02; half-width = 87 ± 14% of baseline, P = 0.1) (Fig. 5D). These findings indicate that Ca channels contribute to multiple synaptic responses at near-threshold potentials. More broadly, they suggest that local EPSPs are sufficient to open voltage-sensitive ion channels in the spine head.

No impact of SK channels on single-spine responses. In many neurons, Ca influx through either NMDA-Rs or Ca channels can open Ca-dependent SK channels (Cai et al. 2004). The resulting hyperpolarization suppresses compound EPSPs, including at pyramidal neurons of the prefrontal cortex (Faber 2010). We generated these EPSPs with a larger theta glass electrode positioned near the basal dendrites (Chalifoux and Carter 2011b) (Fig. 6A). We varied the stimulus duration to evoke EPSPs with one of three amplitudes (small = 1.7 ± 0.2 mV, medium = 4.5 ± 0.4 mV, large = 7.7 ± 0.3 mV; n = 10 cells) (Fig. 6B). At resting potentials, blocking SK channels with wash-in of apamin (0.1 μM) enhanced large EPSPs (amplitude = 127 ± 5% of baseline, P = 0.002; half-width = 133 ± 8% of baseline, P = 0.006) (Fig. 6C). In contrast, apamin had no effect on medium EPSPs (amplitude = 108 ± 5% of baseline, P = 0.2; half-width = 112 ± 7% of baseline, P = 0.2) or small EPSPs (amplitude = 96 ± 12% of baseline, P = 0.9; half-width = 113 ± 8% of baseline, P = 0.2). These findings confirm that SK channels can suppress synaptic re-
Ca channels can function in a negative feedback loop at spines to reduce uEPSPs and synaptic Ca signals (Bloodgood and Sabatini 2007b; Gieselsel and Sabatini 2010; Ngo-Anh et al. 2005). However, at resting potentials we found that wash-in of apamin had no effect on uEPSP amplitude (100 ± 0.97; synaptic Ca signals (89 ± 5% of baseline, P = 0.95; n = 12 spines) or near-threshold potentials (uEPSP amplitude = 110 ± 6% of baseline, P = 0.2; uEPSP half-width = 99 ± 8% of baseline, P = 0.4; synaptic Ca signals = 89 ± 6% of baseline, P = 0.2; n = 12 spines) or near-threshold potentials (uEPSP amplitude = 110 ± 6% of baseline, P = 0.2; uEPSP half-width = 111 ± 12% of baseline, P = 0.6; synaptic Ca signals = 100 ± 8% of baseline, P = 0.4; n = 10 spines) (data not shown). Similarly, with 500 μM fluo-4FF, apamin had no effect at resting potentials (uEPSP amplitude = 111 ± 6% of baseline, P = 0.2; uEPSP half-width = 99 ± 8% of baseline, P = 0.4; synaptic Ca signals = 89 ± 6% of baseline, P = 0.2; n = 12 spines) or near-threshold potentials (uEPSP amplitude = 110 ± 6% of baseline, P = 0.2; uEPSP half-width = 111 ± 12% of baseline, P = 0.6; synaptic Ca signals = 100 ± 8% of baseline, P = 0.4; n = 10 spines) (data not shown). These results indicate that in contrast to pyramidal neurons in the hippocampus, isolated inputs do not engage SK channels at these spines.

One potential complication for these experiments is that Ca buffering by the indicator could prevent the activation of SK channels in spines. To test this possibility, we performed similar recordings using dyes with lower Ca buffering (Yasuda et al. 2004). Using 200 μM fluo-5F, we found that apamin had no effect at resting potentials (uEPSP amplitude = 111 ± 6% of baseline, P = 0.2; uEPSP half-width = 99 ± 8% of baseline, P = 0.4; synaptic Ca signals = 89 ± 6% of baseline, P = 0.2; n = 12 spines) or near-threshold potentials (uEPSP amplitude = 110 ± 6% of baseline, P = 0.2; uEPSP half-width = 111 ± 12% of baseline, P = 0.6; synaptic Ca signals = 100 ± 8% of baseline, P = 0.4; n = 10 spines) (data not shown). These results indicate that in the proximal basal dendrites of these pyramidal neurons local Ca influx does not activate SK channels to shape synaptic responses via a negative feedback loop at single spines.

**Na channels enhance synaptic responses at spines.** The ability of Ca channels to regulate synaptic responses suggested that other voltage-sensitive ion channels could also be engaged

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**Fig. 5. Ca channels enhance single-spine responses.** A: two-photon image of a basal dendrite segment, showing spine (s) and parent dendrite (d). Dotted line indicates line-scan path. B: average somatic action potentials (APs) (left) and AP Ca signals in spines (center) under baseline conditions (black) and after wash-in of the cocktail of Ca channel blockers (red). Thick lines indicate means, and shaded regions indicate ±SE. Arrowheads indicate stimulus time. Right: summary of impact of the Ca channel blockers on AP Ca signal amplitude as % of baseline conditions shown as box plots of median (thick line), interquartile range (box), 10–90% range (whiskers), and single data points (open circles). C: average uEPSPs (left) and synaptic Ca signals (center) evoked at resting potentials under baseline conditions (black) and after wash-in of the Ca channel blockers (red). Right: summary of impact of the Ca channel blockers on uEPSP amplitude, uEPSP half-width, and synaptic Ca signal (uCa) amplitude. D: similar to C at near-threshold potentials. *Significance (P < 0.05).
at individual spines. Voltage-sensitive Na channels are present in the basal dendrites of pyramidal neurons, where they help control local excitability (Nevian et al. 2007). Before studying the influence of these channels on synaptic responses, we first assessed their impact on AP Ca signals at spines. To avoid blocking the somatic AP, we locally applied the Na channel blocker TTX (3 \mu M) from a puffer pipette (Fig. 7A). We found that puffing of TTX reduced AP Ca signals (27 ± 4% of baseline, P < 0.0001; n = 28 spines), with no effect on the somatic AP waveform (amplitude = 98 ± 17% of baseline, P = 0.7; width = 114 ± 27% of baseline, P = 0.9; n = 9 cells) (Fig. 7B). In control experiments, we also locally puffed ACSF to confirm stable baseline conditions, with no effect on AP Ca signals (99 ± 6% of baseline, P = 0.2; n = 12 spines). These results confirm that Na channels are present in the basal dendrites and needed to generate AP Ca signals at spines.

Na channels can augment synaptic responses generated by multiple inputs to other pyramidal neurons (Lipowsky et al. 1996; Magee and Johnston 1995; Stuart and Sakmann 1995). At single spines, these channels can also boost uEPSPs (Araya et al. 2007; Carter et al. 2012) but appear to either reduce synaptic Ca signals (Bloodgood and Sabatini 2007b) or have no effect (Grunditz et al. 2008). At resting potentials, we found that wash-in of TTX (1 \mu M) reduced uEPSP amplitude (82 ±
4% of baseline, $P = 0.001$; $n = 11$ spines) and synaptic Ca signals (73 ± 10% of baseline, $P = 0.001$), with no effect on uEPSP half-width (90 ± 10% of baseline, $P = 0.4$) (Fig. 7C). These results indicate that Na channels can enhance both uEPSPs and synaptic Ca signals at spines.

The impact of Na channels may become even greater with subthreshold depolarization, due to engagement of persistent Na channels by synaptic potentials (Carter et al. 2012; Lipowski et al. 2011; Kampa and Stuart 2006). However, it is unknown whether these channels also regulate synaptic potentials and Ca signals evoked at individual spines. To study the influence of these channels on synaptic responses, we first assessed their impact on AP Ca signals at single spines. In the absence of receptor or channel blockers, we found that wash-in of 4-AP (1 mM) triggered epileptic activity, which was effectively blocked by wash-in of TTX (1 µM). At resting potentials in the vicinity of the somatic membrane, Na channel blocker TTX reduced uEPSP amplitude by 11 ± 3% of baseline, $P = 0.0002$; $n = 9$ cells) (Fig. 8A). These findings indicate that A-type K channels are available in the basal dendrites of these neurons and normally act to suppress AP Ca signals at spines.

We next examined whether A-type K channels are also able to impact synaptic responses at single spines. In the absence of receptor or channel blockers, we found that wash-in of 4-AP (1 mM) triggered epileptic activity, which was effectively blocked by wash-in of TTX (1 µM). At resting potentials in the vicinity of the somatic membrane, Na channel blocker TTX reduced uEPSP amplitude by 11 ± 3% of baseline, $P = 0.0002$; $n = 9$ cells) (Fig. 8A). These findings indicate that A-type K channels are available in the basal dendrites of these neurons and normally act to suppress AP Ca signals at spines.

A-type K channels dampen synaptic responses at spines. Our results indicate that Na and Ca channels enhance distinct components of synaptic responses at dendritic spines. Voltage-sensitive A-type K channels are also present in the dendrites of many pyramidal neurons (Hoffman et al. 1997) and can suppress both compound EPSPs (Cai et al. 2004; Losonczy and Magee 2006) and AP Ca signals (Frick et al. 2003; Gasparini et al. 2011; Kampa and Stuart 2006). However, it is unknown whether these channels also regulate synaptic potentials and Ca signals evoked at individual spines. To study the influence of these channels on synaptic responses, we first assessed their impact on AP Ca signals at single spines. In the absence of receptor or channel blockers, we found that wash-in of 4-AP (1 mM) triggered epileptic activity, which was effectively blocked by wash-in of TTX (1 µM). At resting potentials in the vicinity of the somatic membrane, Na channel blocker TTX reduced uEPSP amplitude by 11 ± 3% of baseline, $P = 0.0002$; $n = 9$ cells) (Fig. 8A). These findings indicate that A-type K channels are available in the basal dendrites of these neurons and normally act to suppress AP Ca signals at spines.

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Fig. 8. A-type K channels dampen single-spine responses. A: average somatic APs (left) and AP Ca signals in spines (center) under baseline conditions (black) and after local puffing of 4-aminopyridine (4-AP) (red). Inset: magnification of voltage trace. Thick lines indicate means, and shaded regions indicate ±SE. Arrowheads indicate stimulus time. Right: summary of impact of 4-AP on AP waveform and AP Ca signal as % of baseline conditions shown as box plots of median (thick line), interquartile range (box), 10–90% range (whiskers), and single data points (open circles). Arrowheads indicate stimulus time. Median (thick line), interquartile range (box), 10–90% range (whiskers), and single data points (open circles).

B: average uEPSPs (left) and synaptic Ca signals (center) evoked at resting potentials under baseline conditions in TTX (black) and after wash-in of 4-AP (red). Right: summary of impact of 4-AP on uEPSP amplitude, uEPSP half-width, and synaptic Ca signal (uCa) amplitude.

C: similar to B at near-threshold potentials. *Significance (P < 0.05).

A-type K channels are potently inactivated by subthreshold depolarization, which may prevent them from influencing synaptic responses at spines (Hoffman et al. 1997). Consistent with this prediction, at near-threshold potentials we found that wash-in of 4-AP (1 mM) no longer increased uEPSP amplitude (103 ± 20% of baseline, P = 0.4; n = 10 spines) or synaptic Ca signals (108 ± 9% of baseline, P = 0.9) and continued to have no effect on uEPSP half-width (105 ± 15% of baseline, P = 0.6) (Fig. 8C). In control experiments, wash-in of ACSF confirmed stable conditions in the presence of TTX, both at resting potentials (uEPSP amplitude = 107 ± 4% of baseline, P = 0.3; uEPSP half-width = 122 ± 11% of baseline, P = 0.3; synaptic Ca signals = 94 ± 2% of baseline, P = 0.1; n = 7 spines) and at near-threshold potentials (uEPSP amplitude = 103 ± 20% of baseline, P = 0.4; uEPSP half-width = 105 ± 15% of baseline, P = 0.6; synaptic Ca signals = 108 ± 9% of baseline, P = 0.9; n = 10 spines). Together, these findings indicate that A-type K channels effectively dampen single-spine responses at rest, but this influence is absent near threshold. In summary, our results indicate how multiple glutamate receptors and ion channels impact synaptic responses at dendritic spines, with their influence strongly regulated by the subthreshold membrane potential.

DISCUSSION

We examined synaptic responses evoked at spines in the basal dendrites of layer 5 pyramidal neurons in the mouse prefrontal cortex. Our findings reveal novel roles for glutamate receptors and voltage-sensitive ion channels in electrical and Ca signaling at individual spines. We found that subthreshold depolarization strongly impacts the contributions of both AMPA-Rs and NMDA-Rs to uEPSPs and synaptic Ca signals. Ca channels also enhance synaptic responses but do not trigger a negative feedback loop mediated by SK channels. Finally, Na channels preferentially enhance responses at near-threshold potentials, whereas A-type K channels suppress them only at resting potentials. Together, these results highlight how the subthreshold membrane potential regulates the contributions of multiple receptors and ion channels at single spines.

Dendritic spines are the first stage for processing of glutamatergic inputs onto cortical pyramidal neurons (Yuste 2011).
We know a great deal about the contributions of different glutamate receptors and ion channels to synaptic responses. However, most studies have focused on responses evoked by multiple inputs onto the dendrites. The compound EPSPs evoked by these inputs are often an order of magnitude larger than at individual spines, and the associated Ca signals are less restricted (Chalifoux and Carter 2011b). Thus the kinds of receptors and channels engaged by multiple inputs can be very different from isolated inputs. It is important to characterize synaptic potentials and Ca signals at spines, because these responses regulate both dendritic excitability and synaptic plasticity (Branco and Hausser 2010; Sjostrom et al. 2008).

To study single spines, we used two-photon uncaging, which bypasses presynaptic release and directly activates glutamate receptors (Matsuzaki et al. 2001). We first calibrated laser power by matching the amplitude of synaptic Ca signals evoked by electrical stimulation and uncaging. We then used a standard power to generate uEPSCs, uEPSPs, and synaptic Ca signals at all analyzed spines. This approach is similar to matching miniature EPSP amplitude (Matsuzaki et al. 2001) but differs from standardizing either uEPSC amplitude or photobleaching at each spine (Bloodgood and Sabatini 2007b).

We found that our approach avoided any photodamage or rundown, allowing us to perform drug wash-ins and conduct pairwise comparisons. However, because we sampled fewer spines we could not perform the population comparisons that become possible with steady-state drug application. These different strategies are complementary, and will continue to be useful in examining single-spine responses.

Most previous studies have assessed single-spine responses at resting membrane potentials, which in cortical pyramidal neurons are tens of millivolts from threshold (Steriade et al. 1993). We found that uEPSPs and synaptic Ca signals at spines strongly depend on the subthreshold membrane potential. uEPSPs maintained their amplitude and became broader near threshold, as found in hippocampal pyramidal neurons and striatal medium spiny neurons (Carter and Sabatini 2004; Carter et al. 2012). While synaptic Ca signals were present at rest, they became larger near threshold, as seen in the striatum (Carter and Sabatini 2004). These findings highlight how subthreshold depolarizations, which occur in functioning circuits (Sanchez-Vives and McCormick 2000; Shu et al. 2003), can regulate both electrical and biochemical signaling at spines.

Our results highlight multiple influences of AMPA-Rs and NMDA-Rs on synaptic responses at spines. As found in other neurons, uEPSPs are primarily mediated by AMPA-Rs and synaptic Ca signals by NMDA-Rs. However, these receptors have additional roles, with AMPA-Rs also enhancing synaptic Ca signals and NMDA-Rs contributing to uEPSPs. The electrogenic role of NMDA-Rs becomes prominent near threshold and helps to stabilize and broaden uEPSPs. This likely reflects the relief of the Mg block of NMDA-Rs, which creates an additional, slow synaptic conductance. The influence of AMPA-Rs is present at both potentials and may provide additional relief of Mg block. This influence is also observed in the hippocampus (Bloodgood et al. 2009), although we did not find two distinct phases of Ca influx. These findings illustrate how both AMPA-Rs and NMDA-Rs contribute to uEPSPs and synaptic Ca signals at spines.

The resistance between a spine and its parent dendrite is high (Bloodgood and Sabatini 2005; Harnett et al. 2012; Svoboda et al. 1996), allowing isolated inputs to depolarize the spine head by tens of millivolts (Palmer and Stuart 2009; Tsay and Yuste 2004). This local depolarization has long been predicted to activate a variety of voltage-sensitive conductances in the spine head (Miller et al. 1985; Perkel and Perkel 1985; Segev and Rall 1988, 1998). Voltage-sensitive Ca channels are engaged by multiple inputs and enhance synaptic responses in dendrites (Gillesen and Alzheimer 1997; Magee et al. 1995; Magee and Johnston 1995; Markram and Sakmann 1994; Miyakawa et al. 1992). These channels are also activated by isolated inputs and contribute to synaptic responses at spines (Denk et al. 1995; Egger et al. 2005; Grunditz et al. 2008; Reid et al. 2001; Schiller et al. 1998). Consistent with these results, we found that Ca channels enhance both uEPSPs and synaptic Ca signals at spines. The influence on uEPSPs only occurred near threshold, which may reflect the types of Ca channels available at these potentials. In contrast, the influence on synaptic Ca signals occurred at both potentials and helps explain the residual Ca signal when NMDA-Rs are blocked.

Recent studies underscore more complex roles for Ca channels in regulating single-spine responses. Ca influx into spines can open Ca-activated SK channels, which hyperpolarize the spine head, enhance Mg block of NMDA-Rs, and ultimately dampen uEPSPs and synaptic Ca signals (Bloodgood and Sabatini 2007b; Giessler and Sabatini 2010; Ngo-Anh et al. 2005). However, we found that blocking Ca channels reduced rather than enhanced synaptic responses at spines of cortical pyramidal neurons. One possibility was that activation of SK channels could be masking even greater effects of Ca channels at spines. We confirmed that SK channels effectively dampen large EPSPs evoked by extracellular stimulation (Faber 2010). However, we found that blocking SK channels had no effect on either uEPSPs or synaptic Ca signals at either membrane potential. Thus Ca channels contribute to synaptic responses in these neurons but do not trigger a negative feedback loop involving SK channels at individual spines.

The inability of SK channels to influence uEPSPs or synaptic Ca signals at spines contrasts with previous studies in the hippocampus. It is unlikely that this reflects smaller synaptic Ca signals in our recordings, as the ΔG/Gsat transients are similar in amplitude and time course. It is also unlikely that our Ca indicator prevented SK channel activation, as similar results were seen with lower buffer capacities. Other explanations include differences in the types of Ca and SK channels or their locations at synapses, spines, and dendrites. Recent results show that SK channels can regulate AP Ca signals in the distal dendrites of cortical pyramidal neurons (Jones and Stuart 2013). To study the impact of subthreshold depolarization from the soma we focused our study on proximal spines, but it will be interesting to explore other dendritic domains in the future. Together, these findings underscore how the interplay of receptors and channels at single spines can vary between and within different types of pyramidal neurons.

In many neurons, multiple inputs onto the dendrites activate voltage-sensitive Na channels to enhance compound EPSPs (Lipowsky et al. 1996; Magee and Johnston 1995; Stuart and Sakmann 1995). We found that Na channels enhance uEPSPs at resting potentials, as observed in other cortical pyramidal neurons (Araya et al. 2007). Na channels also prolong the...
decay of these uEPSPs near threshold, consistent with the activation of persistent Na channels (Carter et al. 2012). Importantly, our results indicate that Na channels also enhance synaptic Ca signals evoked at individual spines. This finding again contrasts with the hippocampus, where Na channels help activate Ca channels that in turn activate SK channels (Bloodgood and Sabatini 2007b). In principle, the impact of Na channels on uEPSPs could occur at activated spines, nearby dendrites, or the cell body. However, the impact of Na channels on synaptic Ca signals suggests a local influence within spines themselves. These findings support the idea that isolated inputs can sufficiently depolarize the spine head to open Na channels.

The ability of Ca and Na channels to shape synaptic responses suggested that voltage-sensitive K channels may be engaged at spines. A-type K channels are present in the dendrites of layer 5 pyramidal neurons in the mouse prefrontal cortex (Acker and Antic 2009; Zhou et al. 2008). In contrast to Na and Ca channels, A-type K channels strongly suppress compound EPSPs evoked by multiple inputs (Cai et al. 2004; Losonczy and Magee 2006). We found that A-type K channels also dampen both uEPSPs and synaptic Ca signals at individual spines. This effect only occurred at resting potentials, consistent with inactivation of these channels near threshold (Hoffman et al. 1997). The impact on uEPSPs could be due to channels in spines or dendrites, as both compartments undergo depolarization. However, the change in synaptic Ca signals again suggests the involvement of A-type channels within the spine head. One caveat is that these experiments were conducted in blockers of Na channels in order to prevent epileptic activity. Suppression by A-type K channels may thus be even greater when Na channels are available to further depolarize the spine head. Similarly, engagement of these channels may be greater in the distal dendrites, where their density is often higher (Hoffman et al. 1997). These findings reveal a novel role for A-type K channels in regulating synaptic potentials and Ca signaling at single spines.

Our results reveal how the subthreshold membrane potential regulates glutamate receptors and ion channels at single spines. At resting potentials, AMPA-Rs evoke uEPSPs and enhance synaptic Ca signals, while generating a large depolarization within the spine head. This depolarization engages Na and Ca channels, which can further enhance uEPSPs or synaptic Ca signals. These effects are balanced by activation of A-type K channels, which dampen synaptic responses at spines. At near-threshold potentials, NMDA-Rs become more prominent, contributing to both synaptic Ca signals and uEPSPs. Na channels also become more engaged to enhance synaptic responses, whereas A-type K channels no longer dampen them. Thus the inactivation of A-type K channels allows other channels to generate prominent nonlinearities at individual spines. While the effects of these channels on uEPSPs often mirror those on compound EPSPs, many of the changes in synaptic Ca signals have not been studied. Interestingly, many of these receptors and channels are also modulated at the level of single spines (Chalfouix and Carter 2010, 2011a; Giessel and Sabatini 2010). In future studies, it will be particularly important to consider how the impact of different neuromodulators depends on the subthreshold membrane potential.

The regulation of uEPSPs and synaptic Ca signals at individual spines has several functional implications. The broadening of uEPSPs at near-threshold potentials may promote interactions between inputs onto multiple spines (Branco and Haussler 2010; Losonczy and Magee 2006). Similarly, the enhancement of synaptic Ca signals may help to regulate Ca-dependent signaling and synaptic plasticity at spines (Bloodgood and Sabatini 2007b; Nimchinsky et al. 2002).

These changes in electrical and biochemical signaling may be particularly important in the prefrontal cortex (Gonzalez-Burgos and Barriouneuveu 2001; Seamans et al. 1997). For example, interactions between receptors and ion channels are thought to contribute to persistent activity (Durstewitz et al. 2000; Wang 2001). Our results suggest that subthreshold depolarization may enhance this activity, while also promoting conditions needed for synaptic plasticity at individual spines. Thus the interactions we have described are likely to contribute to synapse, neuron, and circuit function in the prefrontal cortex.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


