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Influx of extracellular calcium regulates actin-dependent morphological plasticity in dendritic spines

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Abstract

Dendritic spines contain a specialized cytoskeleton composed of dynamic actin filaments capable of producing rapid changes in their motility and morphology. Transient changes in Ca^{2+} levels in the spine cytoplasm have been associated with the modulation of these effects in a variety of ways. To characterize the contribution of Ca^{2+} fluxes originating through different pathways to these phenomena, we used time-lapse imaging of cultured hippocampal neurons expressing GFP-actin to follow the influence of postsynaptic neurotransmitter receptors, voltage-activated Ca^{2+} channels and release from internal Ca^{2+} stores on spine actin dynamics. Stimulation of AMPA receptors produced a rapid blockade of actin-dependent spine motility that was immediately reversible when AMPA was removed. Stimulation of NMDA receptors also blocked spine motility but in this case suppression of actin dynamics was delayed by up to 30 min depending on NMDA concentration and motility was never seen to recover when NMDA was removed. These effects could be mimicked by depolarizing neurons under appropriate circumstances demonstrating the involvement of voltage-activated Ca^{2+} channels in AMPA receptor-mediated effects and the receptor associated Ca^{2+} channel in the effects of NMDA. Caffeine, an agent that releases Ca^{2+} from internal stores, had no immediate effect on spine actin, a result compatible with the lack of caffeine-releasable Ca^{2+} in cultured hippocampal neurons under resting conditions. Blocking internal store function by thapsigargin led to a delayed suppression of spine actin dynamics that was dependent on extracellular Ca^{2+}. Together these results indicate the common involvement of changes in Ca^{2+} levels in modulating actin-dependent effects on dendritic spine motility and morphology through several modes of electrophysiological activation.

Keywords: Excitatory synapse; Glutamate receptor; Hippocampus; Cytoskeleton; Synaptic plasticity; Dendritic spines

1. Introduction

The functional state of excitatory synapses in the central nervous system is closely correlated with the morphology of dendritic spines that form their postsynaptic elements (Harris, 1999; Yuste et al., 2000; Nimchinsky et al., 2002). Over the past several years live cell imaging studies have demonstrated that, whereas most aspects of neuronal morphology are stable over long periods (Kaech et al., 2001; Katz and Mizrahi, 2003), the shapes of dendritic spines can change rapidly (Fischer et al., 1998; Dunaevsky et al., 2001) suggesting a potential mechanism linking morphological and functional plasticity at excitatory synapses in the brain (Halpain, 2000; Matus, 2000; Yuste and Bonhoeffer, 2001). This enhanced morphological plasticity of dendritic spines is correlated with the presence of high concentrations of cytoplasmic actin in the spine cytoplasm coupled with dynamic actin filaments that drive rapid lamellipodia-like surface ruffling of spine heads (Kaech et al., 1997; Roelandse et al., 2003).

Although the mechanisms that regulate the dynamic activity of postsynaptic actin are not well understood, the involvement of Ca^{2+} fluxes is a common feature in studies of activity-dependent changes of spine shape and motility. Time-lapse recordings of hippocampal

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neurons expressing GFP-actin have shown that rapid and reversible blockade of spine actin dynamics following activation of postsynaptic AMPA receptors depends on Ca^{2+} influx via low voltage-activated calcium channels (Fischer et al., 2000) whereas delayed suppression of actin dynamics induced by stimulating postsynaptic NMDA receptors required Ca^{2+} influx through the receptor-associated channel (Ackermann and Matus, 2003). Similarly, fast contraction of spines induced by trains of action potentials was correlated with Ca^{2+} transients in spine heads and was blocked by the actin polymerization antagonist latrunculin-B (Korkotian and Segal, 2001). Conversely, release of Ca^{2+} from internal stores induced by caffeine treatment was reported to cause a rapid increase in the size of existing spines coupled with the growth of new spines (Korkotian and Segal, 1999). Other experiments suggest Ca^{2+} influx via NMDA receptors or voltage-activated channels may differently affect the actin cytoskeleton of dendritic spines compared to other cell compartments (Wu et al., 2001; Furuyashiki et al., 2002).

To directly compare the phenomena described in these diverse studies, we have examined the effects on spine actin dynamics of increases in Ca^{2+} levels originating from different sources. We find a close correlation between the ability of various stimulations to elevate intracellular calcium levels and their effects on spine motility, suggesting that multiple pathways converge in the regulation of dendritic spine motility and morphology.

2. Results

2.1. Different modes of Ca^{2+} entry have similar effects on actin dynamics in dendritic spines

To compare the effects on spine motility of changes in cytoplasmic Ca^{2+} levels from different sources (Fig. 1), we made time-lapse recordings of actin dynamics in dendritic spines of mature hippocampal neurons (>21 days in dissociated cell culture) that had been transfected to express GFP-tagged γ-cytoplasmic actin (GFP-actin). Using epifluorescence or 2-photon laser scanning microscopy time-lapse recordings were made from visual fields containing segments of dendrite with 10–20 spines and projections of z-stacks, each containing three images, 1 μm apart recorded at 20 s intervals, were used to calculate a shape factor for individual spines (Fig. 1B). Fluctuations in shape factor values during the initial phase of each recording indicated that spines are highly motile under resting conditions (Fischer et al., 1998). Suppression of spine dynamics is characterized by a reduction in shape factor variation coupled with a shift in shape factor value

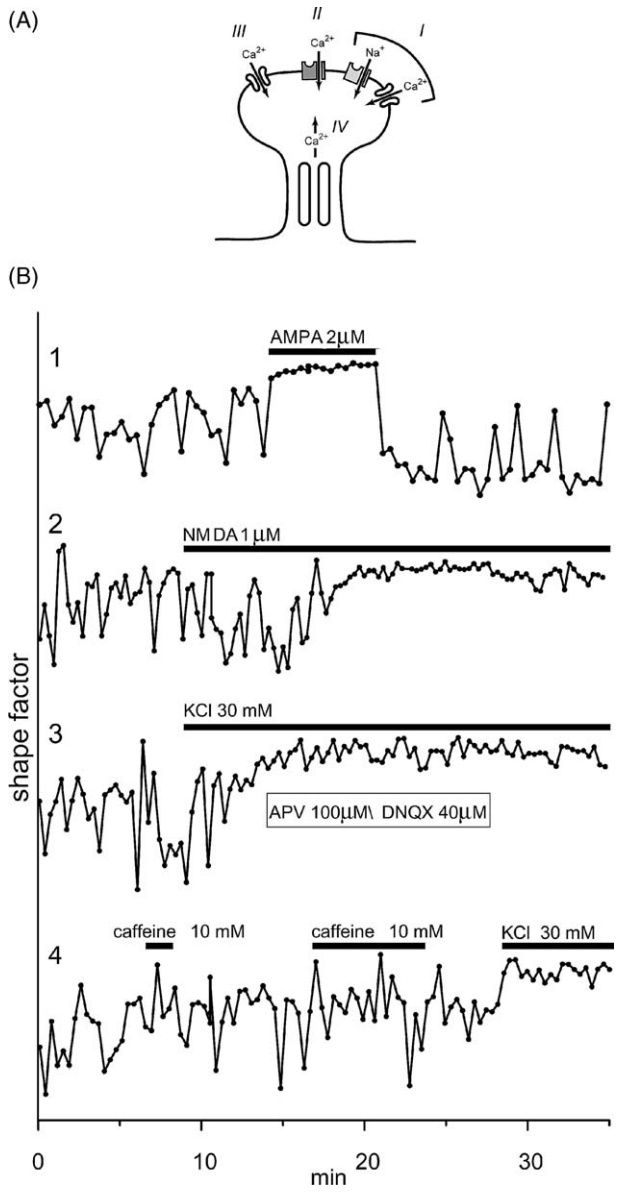

Fig. 1. Inhibition of actin-based spine motility by Ca^{2+} from diverse sources. (A) Diagram showing the four Ca^{2+} sources investigated in this study. I. AMPA receptor mediated opening of voltage-gated Ca^{2+} channels, II. Ca^{2+} entry through NMDA receptors, III. Activation of voltage-gated Ca^{2+} channels via non-synaptic membrane depolarization, IV. Release of Ca^{2+} from internal stores. (B) Shape factor of dendritic spine motility calculated from time-lapse recordings of actin dynamics made using GFP-actin. Rapid fluctuations in shape factor values at the beginning of each recording reflect spine motility under control conditions. Black bars indicate the duration of treatment with various agents as indicated. **1** Exposure to AMPA (2 μM, 5 min) produces rapid suppression of spine motility that is immediately reversible. **2** Addition of NMDA (1 μm) to the medium also inhibits spine motility but with a delay of ~15 min. Motility does not recover when NMDA is washed out. **3** Depolarization with 30 mM KCl blocks spine motility in the presence of the glutamate receptor antagonists APV (100 μM) and DNQX (40 μM). **4** Spine motility is unaffected by application of caffeine to a dendrite either locally for 2 s or 5 min (first bar) or by bath application of 10 mM caffeine (second bar). Subsequent exposure of the same dendrite to 30 mM KCl immediately inhibited spine motility.

towards 1, indicating rounding-up of spine heads (Fischer et al., 2000).

Following exposure to 2 μM AMPA, spine motility ceased completely in less than a minute. A typical example of this behavior is show in Fig. 1B(**1**). The same result was obtained for all spines in segments of dendrite from four independently recorded hippocampal neurons exposed to this concentration of AMPA. The rapid onset of these effects suggests that AMPA receptor activation induces an immediate rearrangement of the spine actin cytoskeleton coupled with blockade of filament dynamics. When AMPA was removed from the cells, spine motility recovered within a minute of starting perfusion with regular medium (Fig. 1B(**1**)) suggesting that the underlying actin-based mechanism is instantaneously reversible. Additional experiments with higher doses of AMPA (3 μM, $n = 6$ and 10 μM, $n = 5$) gave the same result without any detectable differences in either the time course or the reversibility of the effect whereas at lower AMPA concentrations (1 μM, $n = 5$) spine motility was not entirely blocked (data not shown). In their rapidity of onset and reversibility these results are consistent with those of (Fischer et al., 2000) except that in the present series of experiments 2 μM rather than 1 μM agonist was required to completely block spine motility. This difference reflects changes in available culture materials over time that induces small changes in the behaviour of cultured neurons.

Exposing cells to 1 μM NMDA also led to robust stabilization of spine structure but, in contrast to the immediate effect of AMPA, this effect occurred only after a delay of ~15 min following addition of drug-containing medium (Fig. 1B (**2**), $n = 3$). Increasing the concentration of NMDA shortened the delay before spine motility stopped to ~10 min at 2 μM NMDA ($n = 4$) and ~5 min at 5 μM ($n = 4$). At 10 μM NMDA spine motility was blocked without measurable delay ($n = 2$) whereas, at the other end of the concentration range, 0.5 μM NMDA had no detectable inhibitory effect ($n = 2$). Since our extracellular medium contained physiological concentrations of Mg^{2+} (2 mM), NMDA receptors (NMDARs) would remain blocked even after agonist binding under resting conditions. If, however, depolarization were to reach a critical level in any part of a cell, sodium influx through NMDA receptors would produce further depolarization, leading to unblocking of all agonist-bound NMDARs. When it did occur, NMDAR-mediated inhibition of spine motility was always irreversible, even in experiments where, following exposure to 10 μM NMDA for 5 min, time-lapse recordings were continued for >30 min after drug withdrawal ($n = 5$, data not shown).

We next asked whether NMDAR activation and subsequent depolarization are sufficient to arrest spine motility, or if NMDAR-mediated calcium influx is essential to trigger changes in actin dynamics. To distinguish between these possibilities, we tested the effect of NMDA application in media containing different concentrations of extracellular calcium $[Ca^{2+}]_o$. For $[Ca^{2+}]_o = 1$ mM, 5 μM NMDA, which gives robust blockade of spine motility at the regular $[Ca^{2+}]_o$ of 2 mM, had no inhibitory effect ($n = 2$). In nominally Ca^{2+}-free recording medium, even 10 μM NMDA was ineffective in blocking spine motility ($n = 1$). From these experiments, we conclude that calcium influx, but not the depolarization provided by NMDAR activation, triggers the arrest of spine motility.

If intracellular calcium concentration, rather than receptor-specific mechanisms, controls spine motility, activation of voltage-gated Ca^{2+} channels by depolarization would also be expected to block spine motility. Accordingly we exposed cells to 30 mM KCl in the presence of blockers for ionotropic glutamate receptors (APV 100 μM, DNQX 40 μM). Under these conditions, spine motility stopped after a delay of approximately 3 min (Fig. 1B(**3**), $n = 4$), suggesting that intracellular calcium concentration could be the main state variable controlling spine motility irrespective of the mode of Ca^{2+} entry.

2.2. *Activation of internal calcium stores does not affect spine actin dynamics*

Previous work has suggested that release of Ca^{2+} from internal stores plays a role in regulating spine morphology at longer timescales (hours) in cultured hippocampal neurons (Korkotian and Segal, 1999). To assess the potential contribution of this mechanism in regulating fast actin dynamics, we treated neurons with caffeine at 10 mM, a concentration at which it is considered a potent agent for releasing Ca^{2+} from this source. Time-lapse recordings of actin dynamics were made while the drug was applied to dendrites, either by bath application for 5 min or locally by pressure ejection from a micropipette for 2 s. Neither treatment schedule had any detectable effect on actin dynamics in dendritic spines (Fig. 1B(**4**)). This failure could not be attributed to a failure of cellular mechanisms in the dendrite since subsequent exposure of the same cell to 30 mM KCl led to an immediate strong suppression of spine motility equivalent to that seen in other experiments (compare Fig. 1B(**4**) to 1B(**3**)).

As an alternative approach we treated cells with thapsigargin, which blocks reuptake of Ca^{2+} into internal stores, while optically recording actin dynamics in cells transfected with GFP-actin. This revealed an unexpected time-course of action in which the drug initially had no effect on actin dynamics but caused a marked suppression at later times following application. This is demonstrated in Fig. 2 by comparing motility profiles of dendrites derived from 2 min of

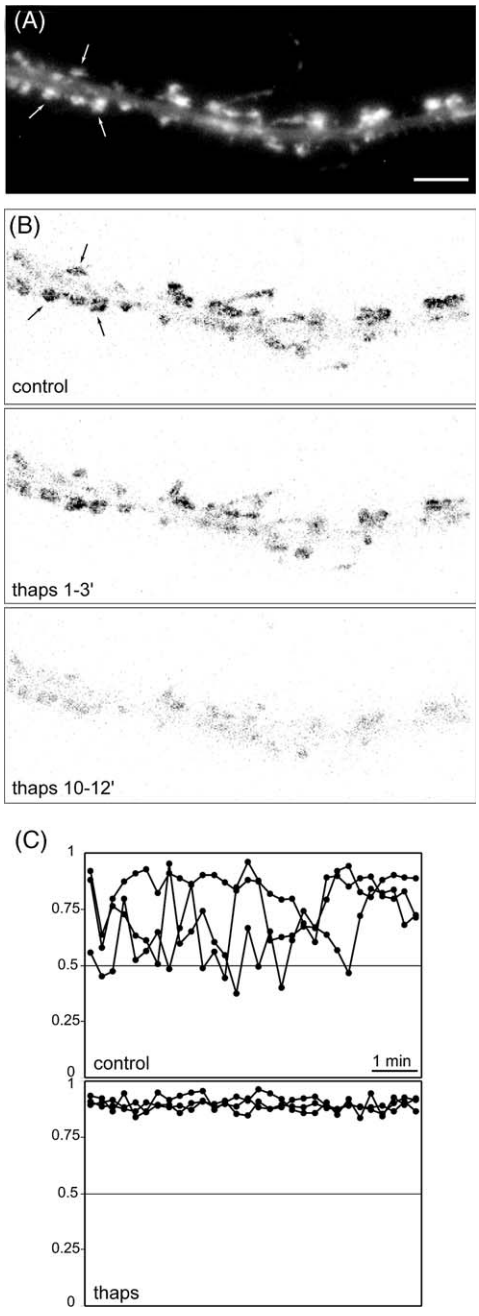

Fig. 2. Delayed suppression of actin dynamics in spines exposed to thapsigargin. (A) GFP-actin fluorescence image of a dendrite segment used for time-lapse recording. Scale bar, 5 μM. (B) Motility profiles derived by summing differences in pixel intensities between successive frames in 2 min excerpts from a time-lapse recording (the darker the image, the higher the motility). The successive panels show, as marked, motility under control conditions, 1 min after the addition of thapsigargin (thaps; 1 μM) and 10 min after thaps was added. (C) Shape factor plots for the three spines indicated by the arrows in (A).

time-lapse recording made at different periods during the experiment. At 3 min after drug application apparent motility did not differ significantly from the control condition (Fig. 2B, panels 1 and 2) but after 10 min motility was blocked (Fig. 2B, bottom panel and C). This effect was highly reproducible as was the delay before the blockade of actin dynamics, which in continuous time-lapse recordings from six independently established cultures occurred 7 min after the addition of thapsigargin.

As a possible source of this delayed effect, we considered store-operated Ca^{2+} entry, the process by which emptying of internal stores induces influx of external Ca^{2+} into the cytoplasm through surface-located channels (Putney, 2003). To test whether this mechanism was responsible for the observed effects, we applied thapsigargin to cells maintained in medium containing a low concentration (0.5 mM) of Ca^{2+} (Fig. 3). As before, actin dynamics were unaffected by thapsigargin after 3 min but in low Ca^{2+} they spine motility remained high even after 12 min (Fig. 3B, panels 1–3). When the medium was then replaced with one containing the regular concentration of Ca^{2+} (2 mM) spine motility was immediately blocked (Fig. 3B, panel 4) indicating that influx of external Ca^{2+} is responsible for the delayed suppression of actin dynamics induced by thapsigargin.

2.3. Differences in Ca^{2+} fluxes from alternative sources underlie their varying effects on spine actin dynamics

Each of the above phenomena implicate changing Ca^{2+} levels in the spine cytoplasm in regulating actin dynamics and hence spine motility. If this is so then the differing effects on actin dynamics of AMPA, which produced an immediate blockade, and caffeine which, despite its putative role as a releaser of Ca^{2+} from internal stores had no immediate effect, might reflect differences in the levels of spine [Ca^{2+}] they induce. To examine this possibility, we analyzed and compared dendritic Ca^{2+} transients evoked by caffeine and AMPA (Fig. 4). Hippocampal neurons were filled with a mixture of the green Ca^{2+}-sensitive dye Fluor5F ("G" in Fig. 4) and the red Ca^{2+}-insensitive dye Alexa Fluor594 ("R" in Fig. 4) and changes in the ratio of G/R fluorescence were measured by 2-photon laser scanning microscopy. Average values, calculated from four consecutive images made before drug application and at peak response during the 2 s of drug application, revealed large differences in spine Ca^{2+} produced by caffeine compared to AMPA (Fig. 4A and B). Application of AMPA (10 μM) produced large changes in fluorescence ratio of between 3 and 23-fold (Fig. 4B) with an average of eightfold (Fig. 4C). We did not see any difference in G/R fluorescence ratio between spines and dendrites, even during the largest calcium transients (Fig. 4D, inset), indicating diffusional equilibrium. This was expected due to the relatively slow time course of agonist application. Bath application of caffeine (10 mM) had no detectable effect on intracellular calcium

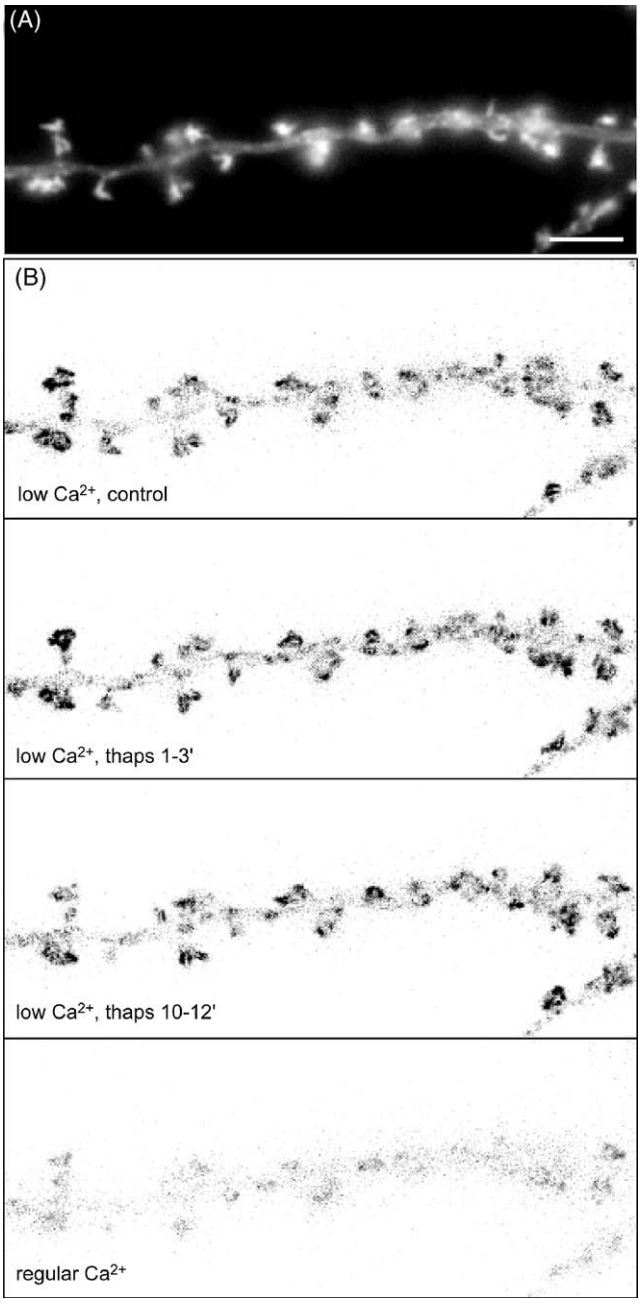

Fig. 3. Thapsigargin blockade of spine motility requires external Ca^{2+}. (A) GFP-actin fluorescence image of a dendrite segment used for time-lapse recording. Scale bar, 5 μm. (B) Motility profiles derived from 2 min time-lapse recordings of the same dendrite showing, as indicated, results for low Ca^{2+} (0.5 mM) medium (control condition), 1 min after addition of thaps (1 μM) in low Ca^{2+}, 10 min after adding thaps still in low external Ca^{2+}, in the continued presence of thaps after readjusting $[Ca^{2+}]_o$ to regular medium (2 mM).

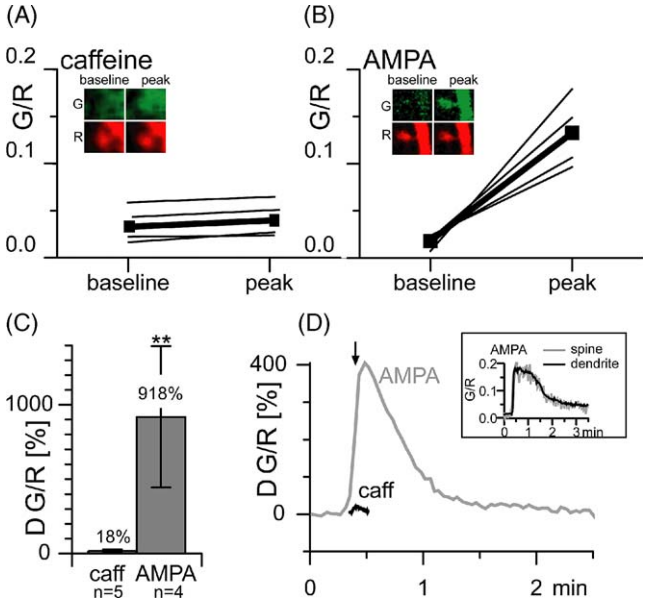

Fig. 4. Calcium transients in spines and dendrites in response to caffeine (caff) and AMPA. (A, B) Recordings from cells filled with the green Ca^{2+}-sensitive dye Fluo5F (green channel, G) and the red Ca^{2+}-insensitive dye Alexa Fluor 594 (red channel, R). Ca^{2+} signals are displayed as the ratio G/R. Drug solutions were applied locally to dendrites for 2 s. Baseline and peak values are each averages of four measurements, before and after drug application, for each cell (caff, $n = 7$; AMPA, $n = 4$). Insets show examples of dendrites with single spines at baseline and peak before and after application of (A) 10 mM caff and (B) 10 μM AMPA. (C) Changes in the G/R ratios (ΔG/R [%]), normalized to baseline values, for all cells in (A) (caff) and (B) (AMPA). (D) Original traces of drug-induced changes in G/R ratio measured at regions of interest in spine or dendrites showing a 51% increase for caffeine compared to a 412% increase for AMPA. Inset: superimposed Ca^{2+} fluorescence ratio measurements

concentrations ($n = 3$, data not shown). To ensure that we were not missing fast calcium transients due to our slow three-dimensional sampling of fluorescence intensity, we performed a second set of caffeine experiments, using local pressure application of caffeine to individual dendrites while sampling the fluorescence at 4 Hz in a single focal plane. We detected small and transient changes in G/R ratio between 5% and 66% with an average of 18% (Fig. 4A, $n = 5$), indicating calcium transients about 45 times smaller in amplitude compared to the AMPA-induced transients. Similar small and transient calcium increases upon caffeine stimulation have been reported by (Korkotian and Segal, 1999), using a high affinity calcium indicator (10 mM OGB1).

3. Discussion

In the present study, we investigated a form of fast spine motility that is caused by continuous remodeling of actin-rich protrusions from the head of the spine and results in rapid changes of the spine outline. We have shown that different experimental conditions, which evoke Ca^{2+} entry from outside the cell, are effective in inhibiting this form of spine motility, such as AMPA receptor-dependent or -independent membrane depolarization or NMDA receptor activation. While application of AMPA led to immediate arrest of

motility, the NMDA effect was delayed in a dose-dependent fashion. This dose-dependent delay is most probably due to the need to build up sufficient post-synaptic depolarization to remove the NMDA receptor channel's Mg^{2+} block and permit calcium influx. NMDA spikes based on regenerative NMDA receptor activation have been described in dendrites of pyramidal cells (Schiller et al., 2000). Our finding that the delay until spine "freezing" is dependent on NMDA concentration, but not the amplitude or the reversibility of the effect, is consistent with the delayed activation of a positive feedback loop by NMDA receptor opening. AMPA receptors, on the other hand, although having a low permeability for calcium ions themselves, lead to immediate depolarization and activation of voltage-gated calcium channels (Fig. 4). It remains to be determined whether molecular pathways become activated downstream of AMPA- and NMDA-receptors activation. The long-lasting NMDA receptor-mediated effects depend on Ca^{2+}-induced changes in the composition of the actin cytoskeleton involving targeting of the small actin-binding protein profilin (Ackermann and Matus, 2003). It is possible that another mechanism involving modification of the spine actin cytoskeleton underlies the transient effects of AMPA receptor activation but the molecules involved have yet to be identified.

3.1. Spine motility and calcium release

The most parsimonious hypothesis to explain the similar effects of different stimulations is to assume intracellular calcium $[Ca^{2+}]_i$ as the main state variable controlling actin polymerization and thus spine motility. It might seem contradictory that application of caffeine, reported to mobilize calcium from intracellular calcium stores via ryanodine receptor activation, did not affect spine dynamics in our experiments (Fig. 1B(**4**)). Compared to AMPA receptor stimulation, however, caffeine stimulation induced only tiny and brief calcium transients in our cells, pointing towards a low filling state of the calcium stores at rest (Fig. 4). Activating calcium release through muscarinic stimulation and IP_3 generation has previously been used to probe the filling state of intracellular calcium stores in cultured hippocampal neurons, with similar results (Irving and Collingridge, 1998). We conclude that duration and amplitude of caffeine-evoked Ca^{2+} transients were not sufficient to trigger inhibition of spine motility in our preparation. Because equilibration of the calcium concentration between spines and dendrite is rapid compared to the time course of our stimulation (Fig. 4, inset), it is unlikely that precise localization of calcium release vs. calcium entry accounts for the different effects on spine motility.

3.2. Spine motility and store operated Ca^{2+}-entry

Given the lack of an effect of store depletion by caffeine application, it was surprising that thapsigargin, a drug leading to slow store depletion by the block of SERCA pumps (sarcoplasmic/endoplasmic reticular Ca^{2+}-ATPase), did cause inhibition of spine motility. The effect was delayed by about ~10 min (Fig. 2), consistent with the time course of store depletion caused by thapsigargin. Importantly, thapsigargin was only effective when the extracellular solution contained Ca^{2+} (Fig. 3). This finding suggests that depletion of internal stores evoked Ca^{2+} influx from outside the cell, a phenomenon known as store-operated calcium entry (SOC).

SOC was first described in non-excitable cells like macrophages, lymphocytes and hepatocytes (Putney, 1986; Parekh and Penner, 1997; Lewis, 1999; Parekh, 2003). The current underlying SOC was termed calcium-release-activated-calcium-current or I_{CRAC} (Hoth and Penner, 1992). I_{CRAC} is mediated by a family of ion channels with high Ca^{2+} selectivity and low single-channel conductance whose molecular identity is still controversial. SOC was also demonstrated in vertebrate neurons (Koizumi et al., 1999Bouron, 2000) where it influences the frequency of spontaneous transmitter release (Savic and Sciancalepore, 1998; Emptage et al., 2001) and might play a role in synaptic plasticity (Baba et al., 2003). Application of thapsigargin to cultured hippocampal neurons bathed in Ca^{2+}-free solution causes only small increases in $[Ca^{2+}]_i$, but re-addition of Ca^{2+} to the external medium leads to pronounced calcium influx (Bouron, 2000; Baba et al., 2003). Our observation that thapsigargin treatment in Ca^{2+}-free solution inhibited spine motility only after reintroducing Ca^{2+} to the external medium is in agreement with these data, supporting the hypothesis that the motility arrest we have observed is triggered each time $[Ca^{2+}]_i$ reaches a threshold level.

Comparing three different pathways for Ca^{2+} entry (voltage-gated calcium channels, NMDA receptor channels, SOC) and calcium release from intracellular stores, we have found no discrepancy between the ability of the various stimuli to raise $[Ca^{2+}]_i$ and their potency in stopping spine motility. It remains possible, however, that microdomains of high calcium concentration rather than bulk calcium are responsible for the inhibition of spine motility reported here. Further experiments are needed to identify the calcium-binding proteins that mediate the calcium-dependent arrest of spine motility and to determine their subcellular localization. Gelsolin, for example, has already been demonstrated to be partially responsible for the activity-dependent stabilization of spines (Star et al., 2002).

Spine motility is a phenomenon critical for structural plasticity in neuronal circuits (Halpain, 2000; Matus,

2000) and it is affected by stimulation protocols that induce LTP and LTD (Ackermann and Matus, 2003). The results presented in this study indicate that spine dynamics in hippocampal neurons can be stabilized not only by voltage-gated Ca^{2+} channels, AMPA and NMDA receptor signaling, but also by store-operated Ca^{2+} entry. Altogether, evidence is accumulating that SOC, besides its potential function in replenishing internal Ca^{2+} stores, can directly affect synaptic function. Especially in excitable cells, SOC could be another highly regulated Ca^{2+} source that is precisely integrated with other Ca^{2+} sources in order to guarantee functional synaptic transmission and plasticity.

4. Methods

4.1. Calcium imaging

We used a custom-built 2-photon laser scanning microscope based on a BX51WI microscope (Olympus Optical, Japan) and a Ti:sapphire laser (Chameleon, Coherent, Scotland) tuned to $\lambda = 810$ nm. Image acquisition was controlled by an open source software package (ScanImage; Pologruto et al., 2003)) written in Matlab (The MathWorks). Fluorescence was detected in epifluorescence (LUMPlan W-IR2 60X 0.9 NA, Olympus) and transfluorescence modes (achromatic aplanatic condenser, 1.4 NA, Olympus) using photomultiplier tubes (R3896, Hamamatsu, Japan). To separate green and red fluorescence, 560 nm dichroic mirrors and 525/50, respectively, 610/75 band pass filters were used (AF Analysentechnik, Germany). Neurons were filled through a patch electrode for >15 min before imaging. We used Fluo5F (Molecular Probes) as a Ca^{2+} indicator dye (0.2 mM, $K_d = 1.26$ μM under physiological conditions, data not shown) in combination with a Ca^{2+} insensitive red fluorophore (Alexa Fluor 594, Molecular Probes). Series of images (256×256 pixel) were acquired at 0.5 or 2.0 Hz. Drugs were applied locally using a Picospritzer III (General Valve). We subtracted the photomultiplier tube dark current from the images and evaluated the ratio of green/red fluorescence intensity (G/R) in a region of interest centred on a spine or a dendrite. This measure is independent of absolute dye concentration and robust to movement artefacts (Yasuda et al., 2004). A single batch of dye mixture was used for all calcium imaging experiments.

4.2. Time-lapse imaging of actin-GFP

Culturing, transfection and time-lapse imaging of actin-GFP expressing hippocampal neurons in dispersed cell culture was performed as previously described (Fischer et al., 1998).

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