Dendritic spines as basic functional units of neuronal integration

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MOST excitatory synaptic connections occur on dendritic spines[1]. Calcium imaging experiments have suggested that spines constitute individual calcium compartments[2,3], but recent results have challenged this idea[4,5]. Using two-photon microscopy[6] to image fluorescence with high resolution in strongly scattering tissue, we measured calcium dynamics in spines from CA1 pyramidal neurons in slices of rat hippocampus. Subthreshold synaptic stimulation and spontaneous synaptic events produced calcium accumulations that were localized to isolated spines, showed stochastic failure, and were abolished by postsynaptic blockers. Single somatic spikes induced fast-peaking calcium accumulation in spines throughout the cell. Pairing of spikes with synaptic stimulation was frequently cooperative, that is, it resulted in supralinear calcium accumulations. We conclude: (1) calcium channels exist in spine heads; (2) action potentials invade the spines; (3) spines are individual calcium compartments; and (4) spines can individually detect the temporal coincidence of pre- and postsynaptic activity, and thus serve as basic functional units of neuronal integration.

Two-photon fluorescence microscopy is particularly well suited for optical imaging of dendritic spines. In contrast to conventional microscopy, two long-wavelength photons are simultaneously absorbed and combine their energies to excite a fluorophore not normally absorbing at such a wavelength[7]. This permits the use of infrared excitation light, which penetrates deeper into highly scattering media like brain tissue[8]. In addition, because the two-photon absorption rate depends quadratically on the density of photons (light intensity), excitation is limited to the focal volume, resulting in reduced photodamage while providing optical sectioning equivalent to a confocal microscope but without any loss of fluorescence light due to a detector pinhole[9]. We imaged secondary and tertiary branches of dendrites in stratum radiatum from 90 CA1 pyramidal neurons filled with the calcium indicator calcium-green-1 by whole-cell perfusion[10,11]. Dendritic spines were clearly resolved down to a depth of 150 μm below the slice surface and could be imaged at high resolution for up to 40 time-

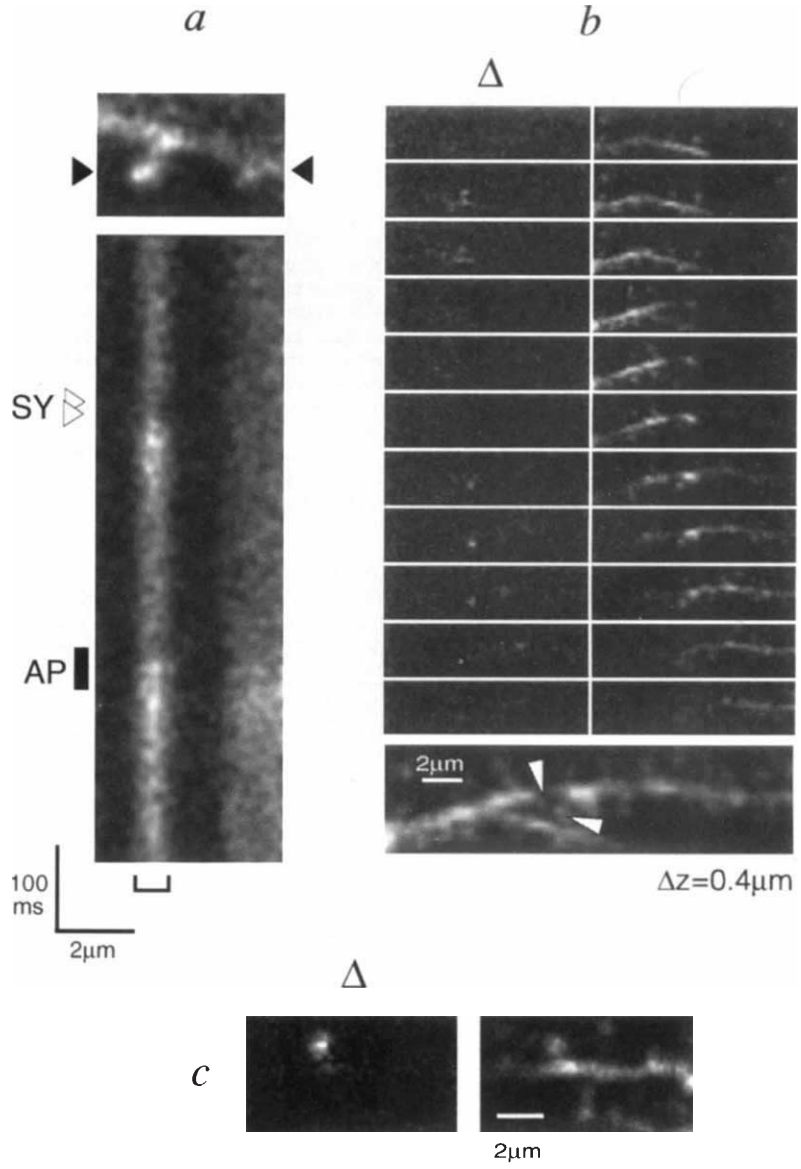

FIG. 1 Subthreshold synaptic stimulation produces calcium accumulations restricted to individual spines. *a*, Fluorescence image (top) and line scan (bottom) of two spines. A line scan is a spatio-temporal image formed by successive scanning of the same line every 2 ms (arrowheads), displayed sequentially. Paired-pulse subthreshold synaptic stimulation (SY; 50 Hz) and a burst of 3 action potentials (AP; 50 Hz) produced transient fluorescence increases due to Ca^{2+} influx. Note that the right spine responds during APs, but not during synaptic stimulation. *b*, Images at different focal planes showing fluorescence intensity at rest (right panels) and difference between subthreshold synaptic stimulation (50 Hz, 100 ms) and rest (Δ, left panels), which highlights those pixels that increase in $[Ca^{2+}]_i$ during the stimulation. Bottom: projection of all images. Note that the only two spines that respond to synaptic stimulation (arrowheads) are in close proximity but located on different branches. *c*, Single spine activation under current clamp (synaptic stimulation: 40 Hz, 125 ms).

METHODS. We used a modified laser scanning microscope (Biorad MRC 600) with a Ti:sapphire laser with 100-fs pulses at 100 MHz (850 nm; Clark Instrumentation) and a 63× 0.9 NA water-immersion objective (Carl Zeiss). Hippocampal slices from 14–30-day-old rats were cut with a vibratome and kept at 24–25 °C in a submerged incubation chamber[30]. After 1–11 h, slices were transferred to a submerged recording chamber at 32 °C. Artificial cerebrospinal fluid (ACSF) contained (in mM): 124 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.23 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, with 95%O_2/5%CO_2. Sometimes APV (Sigma), CNQX (Tocris) or $NiCl_2$ (Sigma) were also included. Recordings[31] were made with a patch-clamp amplifier (EPC-7: List Electronics) operating under voltage- or current-clamp (−70 mV). Electrodes were filled with 135 mM potassium methanesulphonate, 10 mM K-HEPES, 2 mM $MgCl_2$, 0–5 mM Na_2-ATP and 100–500 μM Ca-green; resistances were ~7 MΩ. After break-in we waited 15–30 min before imaging. Electrophysiological signals were recorded using the second input channel of the scanning microscope.

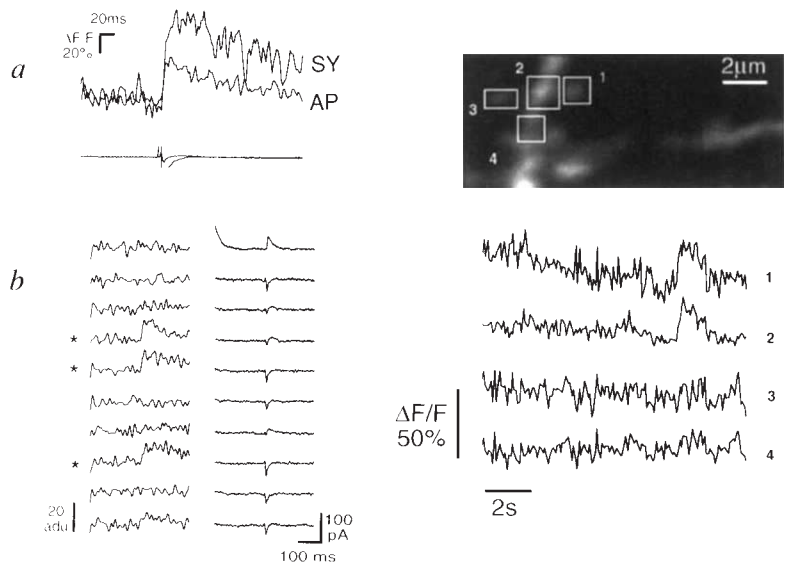

FIG. 2 Synaptic responses show stochastic failures and are similar to spontaneous synaptic events. *a*, Time course of the fluorescence response of a single spine (same as Fig. 1*a*) averaged from 5 trials (out of 19) that showed clear responses to single shocks compared with an average of 19 single-spike-induced responses from the same spine. *b*, Variability of synaptically induced Ca^{2+} accumulations in time-resolved line scans. Left, fluorescence measurements in the same spine in response to single-shock subthreshold synaptic stimulation (adu, digitizing units); right, simultaneous current recordings from the soma. Repeated stimulation with single presynaptic shocks produced variable calcium accumulations that can be classified as successes (asterisks) or failures. Data were smoothed to 8-ms time resolution. *c*, Spontaneous synaptic activity leads to calcium accumulations restricted to a single spine (1) and adjacent dendritic shaft (2), but not in the opposite spine (3) or more distal part of the dendritic shaft (4). Later during the same recording an accumulation restricted to the opposite spine (3) was seen (not shown). Recorded in movie mode (70 by 32 pixels at 13 Hz) in ACSF without Mg^{2+} (nominally), 4 mM Ca^{2+}, and TTX (5 μM).

resolved scans (2 ms per line; 512 lines) without signs of damage (Fig. 1*a*, top).

Focal synaptic stimulation, subthreshold for spike-initiation, induced transient increases of the free-calcium concentration ($[Ca^{2+}]_i$) restricted to spines and short portions of the dendritic shaft close to activated spines (Fig. 1). This was seen under both voltage- and current-clamp recording conditions (Fig. 1*b*, *c*). Spines adjacent to activated spines showed no detectable $[Ca^{2+}]_i$ increases even during trains of subthreshold synaptic stimulation (Fig. 1*b*, *c*). Synaptically induced $[Ca^{2+}]_i$ accumulations rose quickly (Fig. 2*a*), usually to more than half of their peak within 2 ms, the temporal resolution of the measurement.

As we could only find clear synaptically induced calcium responses to subthreshold stimulation when the stimulating electrode was less than 10 μm from the imaged dendrite, we investigated whether the changes measured were due to movement artefacts or to direct activation of voltage-sensitive channels by the stimulating current. We excluded both possibilities by using postsynaptic blockers. The combined perfusion of 2-amino-5-phosphonovaleric acid (APV) (100 μM) and CNQX (20 μM) completely abolished excitatory postsynaptic currents (e.p.s.cs) and calcium accumulation ($n=4$; not shown). As CA1 pyramidal neurons have ~30,000 spines[12] and threshold e.p.s.cs are thought to require only 15–30 quanta[13], our lack of success in finding active spines with remote stimulation can be explained by their scarcity.

Further evidence for a synaptic origin are stochastic failures of calcium to rise after single shock stimulation (Fig. 2*b*). As for e.p.s.cs[14], the probability of failure (normally 0.6–0.8; Fig. 2*b*) dropped to almost zero with paired-pulse stimulation (results not shown).

Experiments on hippocampal cells in culture under conditions of spontaneous transmitter release have suggested that a unitary calcium compartment consists of a roughly 10-μm-long piece of dendrite (including all its spines)[4]. We tested whether the restricted calcium accumulation we found with synaptic stimulation also occurred when observing spontaneous activity (Fig. 2*c*) under high $[Ca^{2+}]$, zero $[Mg^{2+}]$ and tetrodotoxin (TTX), conditions that enhance spontaneous transmitter release[15]. Under these conditions, unlike in cultured neurons[4], calcium accumulation is restricted to individual spines. This chemical compartmentalization is probably the result of the spine geometry, which impedes diffusional exchange between spine head and dendritic shaft[16].

In contrast to the restricted pattern of $[Ca^{2+}]_i$ rise seen with subthreshold synaptic stimulation, a single postsynaptic action potential, triggered by a brief depolarizing pulse (3–10 ms, volt-

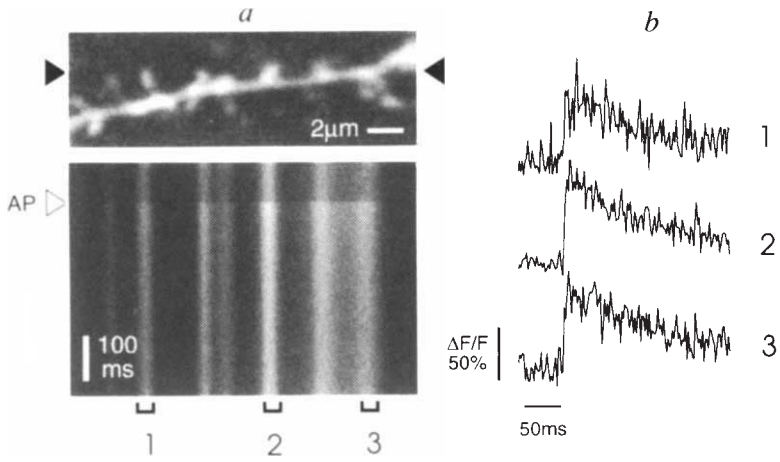

FIG. 3. Somatic action potentials induce calcium accumulations in all spines and dendritic shafts. *a*, Top, fluorescence image of a dendritic segment; bottom, line scan through 6 spines and nearby dendritic shaft (between black arrowheads) during the firing of an action potential in the soma triggered by a 3-ms, 50 mV voltage pulse (white arrowhead). All spines and the dendritic shaft respond simultaneously with significant calcium accumulation. *b*, The response to an action potential in spines and adjacent dendrites is similar. Time course of the fluorescence changes in two spines (1 and 2) and one nearby dendritic area (3) during the single action potential. Note the lack of significant delay between responses.

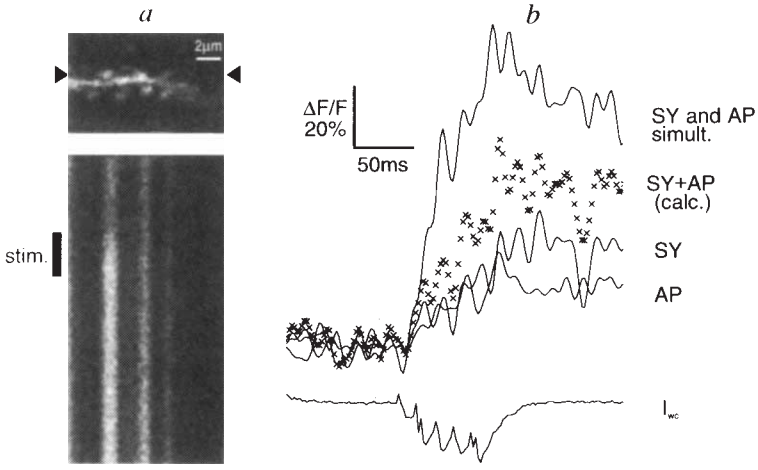

FIG. 4 The association of synaptic stimulation and postsynaptic action potentials produces supralinear calcium accumulations in spines. *a*, Top, fluorescence image of a dendrite; bottom, line scan through three spines (between black arrowheads) during a train of 5 subthreshold e.p.s.cs (75 Hz, stim.). Only the left spine shows calcium accumulations in response to synaptic stimulation (stim.). *b*, Top, time course of the fluorescence response of the left spine to e.p.s.cs (SY), postsynaptic spikes (AP) and their simultaneous combination (SY and AP). Averages of 2 stimulus presentations, which consisted of 5 e.p.s.cs or 5 spikes, produced by five 3-ms, 50 mV somatic voltage pulses, or the combination of both. Also shown for comparison is the calculated sum (SY+AP). In the neighbouring spine, responses to (SY+AP) and (AP) were identical (not shown). Responses were smoothed to 8-ms time resolution. Bottom, current trace during synaptic stimulation (I_{wc}, whole-cell current).

age or current clamp) at the soma, produced a rise of $[Ca^{2+}]_i$ in all imaged spines and dendritic shafts (Fig. 3). Action potentials triggered at the soma invade the dendritic tree of CA1 neurons[17]. Within the time resolution of our measurement (2 ms), we always found $[Ca^{2+}]_i$ to rise simultaneously in spines and adjacent dendritic shaft. These results extend previous measurements with slower time resolution[18]. The occasional spine that did not show spike-induced fluorescence increases always appeared comparatively bright, indicating an already high resting calcium level, presumably saturating the indicator.

The spike-induced calcium influx was due to opening of voltage-sensitive calcium channels (v.s.c.cs) because it was reversibly abolished by perfusion of the v.s.c.c. blocker Ni^{2+} (2 mM; $n=2$), but it remained unchanged ($n=4$) with APV (100 μM) and CNQX (20 μM). Nevertheless, it is possible that calcium released from intracellular stores[19,20] contributed to the later phase of the measured accumulations.

If v.s.c.cs are located only on the dendritic shaft, we estimate, using published diffusion coefficients[21], that it would take at least 30 ms for calcium to diffuse into the spine, even without any diffusional barrier at the spine neck. Therefore, particularly in light of the localization of synaptically induced calcium accumulation, the fast and ubiquitous calcium rise in response to action potentials shows that v.s.c.cs are indeed located on spine heads, as well as on dendritic shafts, and that action potentials invade the spines.

Electrophysiological experiments imply that individual spines might detect the pairing of pre- and postsynaptic activity[22–25]. Therefore we studied the interaction between spikes and synaptic input in single spines. We measured calcium dynamics in the same spine under three conditions: subthreshold synaptic stimulation, spikes, and both together. To avoid failures we used trains of synaptic stimulations. To reduce saturation and nonlinearity of the indicator, we loaded cells with a high (500 μM) concentration of calcium-green. Such a concentration reduces the absolute $[Ca^{2+}]_i$ levels attained, but still permits accurate comparison of influxes[26,27]. We found that in most, but not all, spines the increase in fluorescence (F) resulting from simultaneous stimulation was supralinear, that is, it was larger than the combined responses to the individual stimuli (Fig. 4). Supralinear fluorescence increases were seen under both voltage-clamp (values of 177, 206, 145, 138, 152, 102, 103 and 101%) and current-clamp (134, 68, 106, 102, 110, 112%) recording conditions. As $F([Ca^{2+}])$ is sublinear, or $F([Ca^{2+}]_1+[Ca^{2+}]_2) < F([Ca^{2+}]_1)+F([Ca^{2+}]_2)$, a supralinear fluorescence response implies an even bigger supralinearity of the Ca^{2+} influxes.

Although the mechanism responsible for this $[Ca^{2+}]_i$ supralinearity is still not understood, it is clear that a single spine can use its $[Ca^{2+}]_i$ to register the temporal coincidence of the input and output of the neuron. Cooperative Ca^{2+} accumulation could then be used as a mechanism to target synaptic plasticity[28] or other calcium-dependent forms of chemical computation[29] specifically to individual synapses. □

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