Differential Cholinergic Modulation of Ca\(^{2+}\) Transients Evoked by Backpropagating Action Potentials in Apical and Basal Dendrites of Cortical Pyramidal Neurons

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The effect of the cholinergic agonist carbachol (CCh) on backpropagating action potential (bAP)-evoked Ca\(^{2+}\) transients in distal apical and basal dendrites of layer 2/3 pyramidal neurons in the primary visual cortex of rats was studied using whole cell recordings and confocal Ca\(^{2+}\) imaging. In the presence of CCh (20 \(\mu M\)), initial bAP-evoked Ca\(^{2+}\) transients were followed by large propagating secondary Ca\(^{2+}\) transients that were restricted to proximal apical dendrites \(\leq 40 \mu m\) from the soma. In middle apical dendrites (41–100 \(\mu m\) from the soma), Ca\(^{2+}\) transients evoked by AP bursts at 20 Hz, but not by single APs, were increased by CCh without secondary transients. CCh failed to increase the bAP-evoked Ca\(^{2+}\) transients in distal apical dendrites (101–270 \(\mu m\) from the soma). In contrast, in basal dendrites, CCh increased Ca\(^{2+}\) transients evoked by AP bursts, but not by single APs, and these transients were relatively constant over the entire length of the dendrites. CCh further increased the enhanced bAP-evoked Ca\(^{2+}\) transients in the presence of 4-aminopyridine (200 \(\mu M\)), an A-type K\(^+\) channel blocker, in basal and apical dendrites, except in distal apical dendrites. CCh increased large Ca\(^{2+}\) transients evoked by high-frequency AP bursts in basal dendrites, but not in distal apical dendrites. CCh-induced increase in Ca\(^{2+}\) transients was mediated by InsP\(_3\)-dependent Ca\(^{2+}\)-induced Ca\(^{2+}\)-release. These results suggest that cholinergic stimulation differentially increases the bAP-evoked increase in [Ca\(^{2+}\)]\(_i\) in apical and basal dendrites, which may modulate synaptic activities in a location-dependent manner.

INTRODUCTION

Action potentials (APs) initiated in the axon hillock of cortical pyramidal neurons actively propagate into dendrites (Stuart and Sakmann 1994) and evoke Ca\(^{2+}\) influx in dendrites through voltage-dependent Ca\(^{2+}\) channels (Markram et al. 1995; Spruston et al. 1995). These backpropagating AP (bAP)-evoked dendritic Ca\(^{2+}\) transients are thought to be involved in dendritic excitability, synaptic plasticity, restoration of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) stores, and intracellular metabolic processes (Egorov et al. 1999; Hausser et al. 2001; Magee and Johnston 1997; Waters et al. 2005; Williams and Stuart 2000; Zhou et al. 2005). The amplitude of bAP in apical dendrites of pyramidal neurons decreases with the distance from the soma to the dendrite (Larkum et al. 2007; Spruston et al. 1995). The resulting dendritic Ca\(^{2+}\) transients increase in proximal dendrites and then decrease in distal dendrites (Waters et al. 2003). However, the amplitude of bAP-evoked Ca\(^{2+}\) transients in distal basal dendrites is similar to that in proximal basal dendrites of hippocampal (Hoogland and Saggau 2004) and layer 5 pyramidal neurons (Nevian et al. 2007). In basal dendrites of layer 2/3 pyramidal neurons, the amplitude of bAP-evoked Ca\(^{2+}\) transients in distal dendrites has been reported to be both smaller (Antic 2003) or larger (Cho et al. 2006) than that in proximal dendrites. Synaptic inputs from different areas of the brain are relatively segregated and terminate in isolated dendritic areas (Feldmeyer et al. 2002). Therefore the dendritic location of individual synapses may be critical for the modulation of synaptic activities by the interaction of presynaptic inputs with bAPs and by the resulting Ca\(^{2+}\) influx (Koester and Sakmann 1998; Waters et al. 2003).

Cholinergic stimulation facilitates the backpropagation of AP trains (Tsubokawa and Ross 1997) and increases Ca\(^{2+}\) transients in apical dendrites of hippocampal (Beier and Barish 2000; Nakamura et al. 2000; Tsubokawa and Ross 1997) and neocortical pyramidal neurons (Larkum et al. 2003; Yamamoto et al. 2000). Under cholinergic stimulation, initial bAP-evoked Ca\(^{2+}\) influx induces large secondary propagating Ca\(^{2+}\) waves, which are confined to the thick primary apical dendritic shaft and the soma in hippocampal (Nakamura et al. 2000) and neocortical pyramidal neurons (Larkum et al. 2003; Yamada et al. 2004) and in basolateral amygdala (BLA) neurons (Power and Sah 2007). These large secondary Ca\(^{2+}\) waves are released from Ca\(^{2+}\) stores via inositol 1,4,5-trisphosphate (InsP\(_3\))-receptor activation (Larkum et al. 2003; Nakamura et al. 1999, 2000; Power and Sah 2007). However, little information is available concerning the effect of cholinergic stimulation on Ca\(^{2+}\) transients evoked by bAP in fine dendritic arbors such as distal apical and basal dendrites of cortical pyramidal neurons, where the majority of excitatory synaptic inputs terminate in the primary visual cortex (Larkman 1991). Because the postsynaptic modification of each input is known to depend on its dendritic location (Froemke et al. 2005; Hausser et al. 2001; Letzkus et al. 2006), it is important to more thoroughly under-
stand the dendritic location-dependent cholinergic modulation of bAP-evoked Ca\(^{2+}\) transients.

The present study investigated the location-dependent effects of cholinergic stimulation on bAP-evoked Ca\(^{2+}\) transients in distal apical and basal dendrites of layer 2/3 pyramidal neurons. Results of the present study were previously reported in part in abstract form (Cho et al. 2005).

METHODS

Slice preparation

Coronal slices of primary visual cortex were prepared from Sprague–Dawley rats at postnatal days 21–27. Animal care and surgical procedures were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea and were consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After anesthetization with chloral hydrate (400 mg/kg, administered intraperitoneally), the brains were quickly isolated and then immersed in ice-cold artificial cerebrospinal fluid (ACSF) consisting of (in mM) 125 NaCl, 2.5 KCl, 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 1 CaCl\(_2\), 2 MgSO\(_4\), and 10 D-glucose and aerated with a mixture of 95% O\(_2\)-5% CO\(_2\). Coronal sections (300-μm thick) containing the visual cortex were prepared on a vibr slicer (HM650V, Microm, Walldorf, Germany) and allowed to recover in a submerged slice chamber for 30 min at 37°C. The slices were then maintained at room temperature (22–24°C) in the same ACSF prior to recording. The slices were transferred to the recording chamber and superfused continuously (1–1.5 ml/min) with the same solution, except for the addition of 2 mM CaCl\(_2\) and 1 mM MgSO\(_4\). All experiments were performed at 32–33°C.

Whole cell patch-clamp recording

A standard whole cell patch-clamp technique with a bridge amplifier (BVC-700A, Dagan, Minneapolis, MN) was used to record the membrane potential and to evoke somatic APs. The patch electrodes (4–6 MΩ) pulled from borosilicate glass were filled with a solution containing (in mM) 130 K-gluconate, 10 KCl, 3 Mg-ATP, 10 Na\(_2\)-phosphocreatine, 0.3 Na\(_3\)-GTP, and 10 Hepes (pH 7.25/KOH), supplemented with 50 μM Oregon Green 488 BAPTA-1 (OGB-1; K\(_{90}\) = 170 mM; Molecular Probes, Eugene, OR), 200 μM Fluo-5F (K\(_{d}\) = 2.3 μM, Molecular Probes), or 200 μM Fluo-4FF (K\(_{d}\) = 9.7 μM, Molecular Probes) as a Ca\(^{2+}\) indicator. Pyramidal neurons in layer 2/3 of the primary visual cortex (Paxinos and Watson 1997) were recorded using infrared differential interference contrast video-microscopy with an upright microscope (BX51-WI fitted with a ×40/0.80 NA water-immersion objective; Olympus, Tokyo, Japan) and their regular spiking patterns and the morphology of the bifurcated apical dendrites were confirmed. Typical access resistance was 15–20 MΩ. Membrane potentials were not corrected for an approximately 14-mV junction potential. Command generation, data acquisition, and analyses were performed with the pClamp 9.2 Suite software (Axon Instruments, Foster City, CA). Data were filtered at 5 kHz, sampled at 20 kHz, and saved to a computer hard drive (Pentium PC).

Cells with soma ≤350 μm from the surface of the pia were selected. Cells were used when the resting membrane potential (RMP) was sufficiently negative (less than −68 mV). When carbachol (CCh) was present in the bath solution, some cells were depolarized by 5–7 mV. To eliminate the effect of this membrane depolarization, a hyperpolarizing current was injected via the recording pipette, restoring the RMP. In some experiments, CCh was applied locally near (∼50 μm) the dendritic tree of interest using a patch pipette filled with ACSF. The location of the pipette relative to the dendritic tree of interest and the flow of CCh-containing pipette solution were monitored by using Alexa Fluor 594 (50 μM, Molecular Probes) in the pipette and a 532-nm laser line.

Ca\(^{2+}\) imaging

Fluorescence imaging was performed ≥20 min after obtaining the cells using laser-scanning confocal microscopy (FV-300, Olympus). Light from an argon ion laser (488 nm) was used for illumination and the emitting fluorescence was filtered with a 510-nm long-pass filter. Dendrites were traced from the soma using a fluorescent Ca\(^{2+}\) indicator. The intensity of the laser used for excitation of the indicator was adjusted to minimize phototoxic damage. Dendritic Ca\(^{2+}\) transients evoked by bAPs, which were generated by brief (2- or 10-ms) current injections into the soma, were measured. The fluorescence signals were obtained using either the line-scan (every 1.4–1.6 ms) or area-scan mode (every 17–24 ms). For the line-scanned data, every 10 data points were averaged; for the area-scan data, dendritic areas 10 μm in length were averaged. The distance of the measured area from the soma was calculated from the center of the soma. Fluorescence signals were background-corrected and traces were expressed as the relative change in fluorescence [ΔF/F\(_{0}\) = (F − F\(_{0}\))/F\(_{0}\)], where F\(_{0}\) is the prestimulus fluorescence. The peak amplitude of the Ca\(^{2+}\) transients was determined at the largest ΔF/F\(_{0}\) value of the transients.

Statistics

All data are expressed as the means ± SE. A Student’s t-test and ANOVA with a post hoc Tukey test were used for statistical comparisons. A value of P < 0.05 was considered to be statistically significant.

Drugs

CCh, low-molecular weight heparin, atropine, 4-aminopyridine (4-AP), and all other chemicals, except for ruthenium red (Tocris, Ellisville, MO) and α-dendrotoxin (Alomone Labs, Jerusalem, Israel), were purchased from Sigma (St. Louis, MO).

RESULTS

Cholinergic modulation of bAP-evoked Ca\(^{2+}\) transients along apical dendrites

Whole cell recordings were made from the soma of layer 2/3 pyramidal neurons ≤350 μm from the surface of the pia in the visual cortex. The RMP of the pyramidal neurons was −79.1 ± 0.5 mV and the input resistance was 138 ± 5 MΩ (n = 63). Somatic APs were generated using a positive-step current injection (10 ms, 0.5–1.1 nA) and the resulting dendritic Ca\(^{2+}\) transients evoked by bAPs were measured along the dendrite (Fig. 1A). The peak amplitude of the Ca\(^{2+}\) transients evoked by a single AP and by bursts of three and five APs at 20 Hz increased with the distance from the soma in thick apical dendrites and then declined in distal apical dendrites beyond the point of bifurcation (Fig. 1, B and C). This result was consistent with previous reports from our group (Cho et al. 2006) and from Waters et al. (2003).

When CCh (20 μM) was applied for 5 min at a flow rate of 1 ml/min, AP bursts evoked initial Ca\(^{2+}\) transients followed by large secondary Ca\(^{2+}\) transients in proximal apical dendrites (Fig. 1B). In half of the cells examined (6 of 12 cells), this same CCh-induced pattern of Ca\(^{2+}\) transients was also observed after stimulation with a single AP (Fig. 3A). In middle apical dendrites, CCh treatment increased bAP-evoked initial Ca\(^{2+}\) transients without secondary Ca\(^{2+}\) transients. However,
in distal apical dendrites, bAP-evoked Ca\textsuperscript{2+} transients were not increased in response to CCh. Because the effect of CCh on bAP-evoked Ca\textsuperscript{2+} transients was dependent on dendritic location, apical dendrites were classified into three regions based on the effect of cholinergic stimulation as follows: in proximal dendrites (10–40 μm from the soma) the effect of cholinergic stimulation was in the range observed for the delayed Ca\textsuperscript{2+} transients; middle dendrites (41–100 μm from the soma) did not show any delayed transients, but Ca\textsuperscript{2+} transients evoked by AP bursts were increased compared with the control; and the bAP-evoked Ca\textsuperscript{2+} transients in distal dendrites (>100 μm from the soma) were unaffected by CCh treatment (Fig. 1C). In proximal apical dendrites, the percentage increases in peak amplitude of bAP-evoked Ca\textsuperscript{2+} transients compared with the corresponding control experiments were as follows: 64.3 ± 17.8% for transients evoked by single APs (P < 0.01); 44.7 ± 11.4% after stimulation with a burst of three APs (P < 0.001); and 38.2 ± 6.6% after a burst of five APs (P < 0.001) (Fig. 1D). In middle apical dendrites, CCh increased Ca\textsuperscript{2+} transients evoked by bursts of three and five APs by 16.6 ± 3.5% (P < 0.001) and 24.0 ± 3.1% (P < 0.001) above the control, respectively, whereas CCh failed to increase Ca\textsuperscript{2+} transients evoked by single APs (−2.9 ± 5.3%). However, in distal apical dendrites, CCh failed to increase Ca\textsuperscript{2+} transients evoked by either single APs (−7.2 ± 3.6%) or bursts (three APs: −3.7 ± 3.4%; five APs: −4.6 ± 2.9%) relative to the controls.

Additional analysis was performed on the extent of the effect of CCh in individual cells showing the dendrite up to about 150 μm from the soma with an area scan (Fig. 2). The number of APs was related to the extent of the effect of CCh on bAP-evoked Ca\textsuperscript{2+} transients (single AP: 44 ± 3 μm from the soma; three APs: 78 ± 5 μm; and five APs: 90 ± 4 μm, n = 10), consistent with Fig. 1C. To further investigate the effect of the amount of Ca\textsuperscript{2+} influx on the spatial profile of cholinergic
enhancement of Ca\(^{2+}\) transients, 4-AP (an A-type K\(^+\) channel blocker) was applied to enhance bAP-evoked Ca\(^{2+}\) transients. A bath application of 4-AP (1 mM) enhanced the bAP-evoked Ca\(^{2+}\) transients evoked by single APs and AP bursts in the proximal and middle apical dendrites (Fig. 2B). The CCh-induced increase in 4-AP–enhanced Ca\(^{2+}\) transients extended to slightly more distal dendrites (five APs: 105 ± 5 \(\mu\)m from the soma, \(P < 0.05, n = 8\)). The extent of the Ca\(^{2+}\) increase was best correlated with the distance of bifurcation from the soma (\(r^2 = 0.874\)). Therefore the CCh-induced increase in bAP-evoked Ca\(^{2+}\) transients was primarily dependent on the distance from the soma and was influenced by the amount of the Ca\(^{2+}\) influx.

The effects of CCh on proximal and middle apical dendrites were completely abolished by the application of 10 \(\mu\)M atropine, a muscarinic acetylcholine receptor (mAChR) antagonist, in the presence of CCh (\(n = 10\)) (Fig. 1B). The effect of CCh was completely reversible on wash-out with normal ACSF (\(n = 11\), data not shown). In this study, the application of CCh induced slight depolarization in some cells. To minimize the effects of CCh-induced depolarization, RMP was maintained at pre-CCh-treatment levels with a continuous negative current injection into the soma. CCh application did not affect the shape or parameters of somatic APs (AP threshold: \(-42.8 \pm 1.4\) mV before CCh, \(-43.4 \pm 1.5\) mV after CCh, \(P = 0.89\); AP amplitude: \(67.7 \pm 0.9\) mV before CCh, \(66.1 \pm 1.0\) mV after CCh, \(P = 0.47\); AP width at half-maximal amplitude: \(1.3 \pm 0.1\) ms before CCh, \(1.3 \pm 0.1\) ms after CCh, \(P = 0.94; n = 7\)).

**Mechanism of cholinergic modulation of bAP-evoked Ca\(^{2+}\) transients in apical dendrites**

The synergistic activation of the mAChR with a bAP-evoked Ca\(^{2+}\) influx causes release of Ca\(^{2+}\) from InsP\(_3\)-sensitive Ca\(^{2+}\) stores in proximal apical dendrites and in the soma of neocortical and hippocampal pyramidal neurons (Nakamura et al. 2000; Power and Sah 2002; Yamada et al. 2004). Thus experiments were conducted to determine whether these mechanisms are also responsible for the increase in bAP-evoked Ca\(^{2+}\) transients in middle apical dendrites, which are devoid of secondary Ca\(^{2+}\) transients. The CCh-induced increase in initial Ca\(^{2+}\) transients in middle apical dendrites, as well as secondary Ca\(^{2+}\) transients in proximal apical dendrites, was not observed when the solution in the pipette contained heparin (1 mg/ml) (Fig. 3A). The average percentage increases in Ca\(^{2+}\) transients evoked by five APs in proximal and middle apical dendrites were \(-1.1 \pm 1.6\% (n = 6)\) and \(-1.1 \pm 3.3\% (n = 5)\) relative to the control, respectively (Fig. 3B). Meanwhile, the application of heparin did not change the amplitude of bAP-evoked Ca\(^{2+}\) transients in the absence of CCh (data not shown). Thus the CCh-induced increases in bAP-evoked Ca\(^{2+}\) transients were completely blocked by the InsP\(_3\)-receptor inhibitor heparin both in middle and in proximal apical dendrites.

To examine the possible involvement of the ryanodine receptor, which is another Ca\(^{2+}\)-release channel, ruthenium red (50 \(\mu\)M) was added to the solution in the pipette. CCh induced secondary Ca\(^{2+}\) transients in proximal apical dendrites and increased initial Ca\(^{2+}\) transients in middle apical dendrites in

**FIG. 2.** Spatial profile of cholinergic increase in Ca\(^{2+}\) transients in the presence of 4-aminopyridine (4-AP). A: spatial profile of cholinergic increases in Ca\(^{2+}\) transients evoked by bAP. Cells showing dendritic structure ≤ 150 \(\mu\)m using an area scan were included in the analysis. Either OGB-1 (50 \(\mu\)M, \(n = 5\)) or Fluo-5F (200 \(\mu\)M, \(n = 5\)) was used as a Ca\(^{2+}\) dye. Gray bars and lines afterward represent the main apical trunk before the main bifurcation and the other parts of the whole dendritic trees, respectively. Symbols denote the most distal location where the analyzed area showed an apparent Ca\(^{2+}\) increase. The panels on the right show a representative cell (asterisk in the left panel) and Ca\(^{2+}\) transients measured at the indicated dendritic area (81 \(\mu\)m from the soma) with area-scanned fluorescent images. CCh (20 \(\mu\)M, gray traces) increased only Ca\(^{2+}\) transients evoked by bursts of 5 APs at this location (black traces). B: spatial profile of cholinergic increase in 4-AP–enhanced Ca\(^{2+}\) transients. Fluo-5F (200 \(\mu\)M) was used as a Ca\(^{2+}\) dye. The panel on the right shows representative Ca\(^{2+}\) transients analyzed at 87 \(\mu\)m from the soma, in a cell indicated with an asterisk in the left panel. 4-AP (1 mM, black traces) enhanced the Ca\(^{2+}\) transients evoked by bAPs (dotted traces). CCh further increased Ca\(^{2+}\) transients evoked by single AP and AP bursts at this location (gray traces). The scale bars in the area-scanned images represent 20 \(\mu\)m.
tent with previous reports that, in apical dendrites, the CCh-induced enhancement of bAP-evoked Ca\textsuperscript{2+} transients is due to the release of Ca\textsuperscript{2+} from intracellular stores mediated by InsP\textsubscript{3} receptors, but not by ryanodine receptors (Nakamura et al. 1999; Yamamoto et al. 2000). Power and Sah (2007) also reported that the same mechanism is responsible for the increase in initial Ca\textsuperscript{2+} transients evoked by bAPs in BLA neurons. Thus the results of the present study indicate that InsP\textsubscript{3}-dependent Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) is responsible for the increases in bAP-evoked Ca\textsuperscript{2+} transients in middle apical dendrites as well as for secondary Ca\textsuperscript{2+} transients in proximal apical dendrites.

**Cholinergic modulation of bAP-evoked Ca\textsuperscript{2+} transients in basal dendrites**

The amplitude of the Ca\textsuperscript{2+} transients increased with the distance from the soma along basal dendrites when the cells were stimulated with AP bursts \([F_{(5,57)} = 3.29, P < 0.05\) for three APs; \(F_{(5,72)} = 5.18, P < 0.001\) for five APs] (Fig. 4C), consistent with our previous report (Cho et al. 2006). On CCh application, delayed secondary Ca\textsuperscript{2+} transients were observed in only a few cells (8 of 51 cells), which were located within about 20 \(\mu\text{m}\) of the soma, in basal dendrites (data not shown). Whereas CCh failed to increase Ca\textsuperscript{2+} transients evoked by single APs, the peak amplitudes of Ca\textsuperscript{2+} transients evoked by AP bursts were enhanced by CCh in most regions of basal dendrites (Fig. 4), similar to the response observed in middle apical dendrites. The effects of CCh in basal dendrites were abolished by addition of atropine to the bath (Fig. 4B) and were reversible on wash-out with normal ACSF (data not shown). Basal dendrites were also classified into two regions for quantitative analysis (Fig. 4B). In proximal basal dendrites (10 – 60 \(\mu\text{m}\) from the soma), CCh increased Ca\textsuperscript{2+} transients evoked by AP bursts (23.1 \(\pm\) 6.6\%, \(P < 0.001\) for three APs and 21.7 \(\pm\) 2.9\%, \(P < 0.001\) for five APs), but not by single APs (3.0 \(\pm\) 3.5\%, \(P = 0.94\)). In distal basal dendrites (61–130 \(\mu\text{m}\) from the soma), CCh increased only burst-evoked Ca\textsuperscript{2+} transients relative to the control as follows: 11.1 \(\pm\) 2.5\% for three APs (\(P < 0.05\) and 17.7 \(\pm\) 2.3\% for five APs (\(P < 0.001\)). These values were similar to those obtained in proximal basal dendrites. These results show that the CCh-induced increases in Ca\textsuperscript{2+} transients evoked by AP bursts occur in all regions of basal dendrites, in contrast to the location-dependent response observed in apical dendrites.

It is possible that addition of CCh to the solution that bathes the cells, as well as repeated stimulation of the cells, may deplete calcium stores. However, in this study no noticeable changes in baseline Ca\textsuperscript{2+} levels were observed during the bath application of CCh at a concentration of 20 \(\mu\text{M}\). The amplitude of Ca\textsuperscript{2+} transients after coapplication of CCh and atropine or after CCh wash-out was not different from the control. In addition, the increases in bAP-evoked Ca\textsuperscript{2+} transients by local application of CCh were not different from those induced by bath application of CCh in apical and basal dendrites (see Supplemental Fig. S1).

1 The online version of this article contains supplemental data.
cholinergic stimulation alone in hippocampal pyramidal (Power and Sah 2002) and BLA neurons (Power and Sah 2007). Cellular distribution of mAChR and InsP3 receptors may be responsible for the difference in cholinergic receptor-mediated Ca2+ response between these neurons.

**Mechanism of cholinergic modulation on bAP-evoked Ca2+ transients in basal dendrites**

The role of InsP3 receptors in the CCh-induced increases in bAP-evoked Ca2+ transients in basal dendrites was investigated (Fig. 5). When heparin was added to the pipette solution (1 mg/ml), CCh failed to increase bAP-evoked Ca2+ transients after five APs in proximal (1.1 ± 2.0% relative to the control, n = 5) and distal (0.5 ± 3.0% relative to the control) basal dendrites, respectively. However, when ruthenium red (50 μM) was added to the solution in the pipette, CCh increased the Ca2+ transients evoked by five APs 18.9 ± 7.5 and 12.1 ± 1.9% in proximal and distal basal dendrites, respectively (n = 5). These values were not different from those obtained in the control experiment without heparin or ruthenium red. These results indicate that the CCh-induced increase in bAP-evoked Ca2+ transients in basal dendrites is mediated by InsP3 receptors, but not by ryanodine receptors. Therefore the mechanism of intracellular Ca2+ release by cholinergic stimulation does not differ between basal and apical dendrites.

**Effect of CCh on enhanced bAP-evoked Ca2+ transients**

The increases in Ca2+ transients induced by InsP3-dependent CICR were related to the amplitude of the bAP-evoked Ca2+ influx (Figs. 1C and 2). Therefore the relatively small increase in the [Ca2+]i peak evoked by bAPs may be insufficient to induce Ca2+ release from stores during CCh application in distal apical dendrites (Nakamura et al. 2000; Power and Sah 2007). To test this possibility, 4-AP was added to the bath solution to enhance bAP-evoked Ca2+ transients in distal apical dendrites. Fluo-5F (200 μM) was used to prevent saturation of the fluorescence signal. Because apparent increases in Ca2+ transients were observed at 100 μM 4-AP in distal apical dendrites (206 ± 14 μm from the soma, n = 7) and the EC50 of bAP-evoked Ca2+ transients was about 200 μM (see Supplemental Fig. S2), 200 μM 4-AP was used to evoke large Ca2+ transients with reduced background excitation of the slice. With the application of 4-AP, Ca2+ transients evoked by five APs were enhanced by 90–450% (256.4 ± 67.4%, n = 7, P < 0.01; mean distance: 198 ± 13 μm from the soma) relative to the control (Fig. 6A). However, CCh (20 μM)
cause α-dendrotoxin (250 nM), a D-type K⁺ channel blocker, did not change Ca²⁺ transients in distal apical dendrites (221 ± 13 μm from the soma, n = 8) in this experiment (see Supplemental Fig. S3), 4-AP (200 μM) enhanced dendritic Ca²⁺ transients via inhibition of A-type K⁺ channels in distal apical dendrites. The EC₅₀ of 4-AP on the Ca²⁺ transients in distal apical dendrites (~200 μM) in the present experiment was much smaller than the IC₅₀ of 4-AP on the A-type K⁺ channels (1.4–4.2 mM), which were measured by direct electrophysiological recording (Hoffman et al. 1997; Korngreen and Sakmann 2000). The accumulating effect of 4-AP on the back-propagation of somatic APs en route to the distal dendritic location might explain these discrepancies. These results are consistent with a report that A-type K⁺ channels are involved.

FIG. 5. InsP₃-dependent Ca²⁺-induced Ca²⁺ release by Ch in basal dendrites. A: traces for the control experiment were obtained by the intracellular application of heparin (1 mg/ml) or ruthenium red (50 μM) (shown in black). The Ch-induced (20 μM, shown in gray) increase in bAP-evoked Ca²⁺ transients was blocked by heparin in proximal (30 μm from the soma) and distal (70 μm from the soma) basal dendrites. However, the Ch-induced increase in bAP-evoked Ca²⁺ transients was not blocked by ruthenium red in proximal (15 μm from the soma) and distal (62 μm from the soma) basal dendrites. B: summary of the effects of heparin and ruthenium red on the Ch-induced increase in bAP-evoked Ca²⁺ transients in proximal (21 ± 6 μm from the soma, n = 5, and 28 ± 3 μm from the soma, n = 3, respectively) and distal basal dendrites (72 ± 8 μm from the soma, n = 5, and 74 ± 7 μm from the soma, n = 5, respectively). Data are average increases relative to the control in response to Ch treatment in the presence of heparin or ruthenium red. Ch data were taken from Fig. 4D for comparison. *P < 0.05 vs. Ch.

FIG. 6. Effect of Ch on enhanced bAP-evoked Ca²⁺ transients by 4-AP in distal apical and basal dendrites. A: effect of Ch on enhanced Ca²⁺ transients in distal apical dendrites. Fluo-5F (200 μM) was used to prevent saturation of the fluorescence signal. The left panel shows representative traces of Ca²⁺ transients in an apical dendrite (210 μm from the soma) evoked by 5 APs at 20 Hz in response to the following treatments: normal artificial cerebrospinal fluid (ACSF; Control, shown in black); 4-AP (shown in green); 4-AP and Ch (20 μM) (shown in red); and after wash-out of both drugs (shown in blue). Saturated Ca²⁺ transients were evoked by high-frequency APs (5 APs at 100 Hz, dashed trace). The right panel shows a summary of the effect of 4-AP and Ch in distal apical dendrites (198 ± 13 μm from the soma, n = 7). B: effect of Ch on enhanced Ca²⁺ transients in distal basal dendrites. The left panel shows representative traces of Ca²⁺ transients in a basal dendrite (74 μm from the soma) evoked by 5 APs at 20 Hz in response to the following treatments: normal ACSF (Control, shown in black); 4-AP (shown in green); 4-AP and Ch (shown in red); and after wash-out of both drugs (shown in blue). The right panel shows a summary of the effect of 4-AP and Ch in distal basal dendrites (198 ± 13 μm from the soma, n = 7).
in the backpropagation of somatic APs in basal dendrites of layer 5 pyramidal neurons (Nevian et al. 2007). In summary, CCh further increased 4-AP–enhanced Ca\(^{2+}\) transients in whole dendritic trees, except for distal apical dendrites of layer 2/3 pyramidal neurons in the visual cortex.

**Effect of CCh on high-frequency bAP-evoked Ca\(^{2+}\) transients**

The effects of CCh on Ca\(^{2+}\) transients evoked by high-frequency AP bursts on fine distal apical and basal dendrites were investigated in the next experiment. In distal apical dendrites, increasing Ca\(^{2+}\) transient amplitudes were evoked by increasing the frequency of the three APs ≤100 Hz (Fig. 7A). However, a burst of three APs at 133 Hz evoked smaller-amplitude Ca\(^{2+}\) transients than three APs at 100 Hz, suggesting that bursts at frequencies >100 Hz do not efficiently propagate to the fine distal apical dendrites. The normalized ratio of peak Ca\(^{2+}\) transients evoked by bursts of three APs at 100 Hz relative to those evoked by single APs showed a supralinear increase in Ca\(^{2+}\) transients in distal apical dendrites (5.3 ± 1.0, 186 ± 7 μm from the soma, n = 9) (Fig. 7C). CCh failed to increase the supralinear Ca\(^{2+}\) transients evoked by bursts of three APs at high frequencies (P = 0.45) (Fig. 7A). In distal basal dendrites, the increase in Ca\(^{2+}\) transients was linear (≤133 Hz and 110 μm from the soma; data not shown), but was enhanced by CCh application (Fig. 7B). Supralinear Ca\(^{2+}\) transients in fine distal apical dendrites in layer 2/3 pyramidal neurons were evoked by additional dendritic electrogenesis above the critical frequency (Larkum et al. 2007). In this experiment, however, the increase in Ca\(^{2+}\) transients in basal dendrites was linear, which is different from the supralinear increase in layer 5 pyramidal neurons (Kampa and Stuart 2006). In summary, the differential effect of CCh on bAP-evoked dendritic Ca\(^{2+}\) transients in basal and apical dendrites was not dependent on AP frequency.

**DISCUSSION**

The present study investigated the cholinergic modulation of bAP-evoked dendritic Ca\(^{2+}\) transients in distal apical and basal dendrites of layer 2/3 pyramidal neurons in the rat primary visual cortex. CCh failed to increase high-frequency bAP-evoked supralinear Ca\(^{2+}\) transients in distal apical dendrites. A: effect of CCh on supralinear Ca\(^{2+}\) transients in distal apical dendrites. Fluo-4FF (200 μM) was used as a fluorescent dye. The inset shows the dendritic location where Ca\(^{2+}\) transients were measured (175 μm from the soma). The scale bar is 100 μm. The left and middle panels show representative traces of Ca\(^{2+}\) transients evoked by 3 APs at 20 and 100 Hz (shown in black), respectively, and the effect of CCh (shown in gray). The right panel shows the summary of the effect of CCh on Ca\(^{2+}\) transients evoked by 3 APs at 20 to 133 Hz in distal apical dendrites. B: effect of CCh on large Ca\(^{2+}\) transients in distal basal dendrites. The inset shows the dendritic location where Ca\(^{2+}\) transients were measured (105 μm from the soma). The scale bar is 50 μm. The left and middle panels show representative traces of Ca\(^{2+}\) transients evoked by 3 APs at 20 and 133 Hz (shown in black), respectively, and the effect of CCh (shown in gray). The right panel shows the summary of the effect of CCh on Ca\(^{2+}\) transients evoked by 3 APs at 20 to 133 Hz in distal basal dendrites. C: normalized ratio of Ca\(^{2+}\) transients evoked by 3 APs relative to a single AP at different frequencies. Three APs at 100 Hz evoked supralinear Ca\(^{2+}\) transients in distal apical dendrites.
visual cortex. The major findings are as follows: 1) cholinergic stimulation did not affect single AP- or burst-evoked dendritic Ca$^{2+}$ transients in distal apical dendrites located 101–270 µm from the soma, regardless of the AP frequency used, ≥133 Hz; 2) in contrast to apical dendrites, cholinergic stimulation increased Ca$^{2+}$ transients evoked by AP bursts, but not by single APs, in all basal dendrites; and 3) the mechanism of the cholinergic-induced increase in Ca$^{2+}$ transients involves the release of Ca$^{2+}$ from intracellular stores via InsP$_3$-dependent CICR in both apical and basal dendrites.

Location dependence of InsP$_3$-dependent CICR

The influx of extracellular Ca$^{2+}$ induces InsP$_3$-dependent CICR under the stimulation of generating subthreshold InsP$_3$, at which InsP$_3$ alone induces no Ca$^{2+}$ release from the InsP$_3$-sensitive store (Larkum et al. 2003; Nakamura et al. 2000; Power and Sah 2002; Yamamoto et al. 2000). Because this Ca$^{2+}$ release is from InsP$_3$-sensitive stores, it appears to depend primarily on the density of the InsP$_3$ receptor (InsP$_3$R) and on the capacity of the store. Thus there is a correlation between the distribution of InsP$_3$R1 (Hertle and Yeckel 2007; Sharp et al. 1993) and the spatial profile of the propagating Ca$^{2+}$ wave evoked by a combined increase in InsP$_3$ and intracellular Ca$^{2+}$ (Hagenston et al. 2008; Nakamura et al. 2002) in the soma and proximal apical dendrites. Moreover, the propagating Ca$^{2+}$ wave starts at a branching point (Nakamura et al. 2002) where InsP$_3$R1 immunoreactivity is highly clustered (Hertle and Yeckel 2007). The spatial profile of the CCh-induced InsP$_3$-dependent CICR along the apical dendritic tree to the distal part (±270 µm from the soma) in the present study is consistent with the distribution of InsP$_3$R1 in the hippocampal pyramidal neurons (Hertle and Yeckel 2007), supporting the idea that the density of InsP$_3$R is crucial for InsP$_3$-dependent CICR in pyramidal neurons. Because Ca$^{2+}$ acts as a coagonist on InsP$_3$R1 (Bezprozvanny et al. 1991), the magnitude of the Ca$^{2+}$ influx is also responsible for the extent of InsP$_3$-dependent CICR, as shown in the present (Fig. 2) and in previous (Larkum et al. 2003) studies. However, a certain level of InsP$_3$R and Ca$^{2+}$ release pools seems to be a prerequisite for the Ca$^{2+}$ release from InsP$_3$-sensitive stores because a high increase in [Ca$^{2+}$], did not induce Ca$^{2+}$ release in the fine distal apical dendrites in the presence of CCh and trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD, 50 µM, n = 4; data not shown), a metabotropic glutamate receptor agonist. In addition, the organization of InsP$_3$ signaling microdomains (Delmas et al. 2002; Jacob et al. 2005) and different affinities of InsP$_3$R subtypes for InsP$_3$ (Hagar et al. 1998; Wojcikiewicz and Luo 1998) might also be responsible for the differential effects of CCh along the apical dendritic trees.

In the present study, we observed CCh-induced InsP$_3$-dependent CICR in young animals (3 wk old). It is known that InsP$_3$R1 immunoreactivity becomes more evenly distributed along the apical dendritic tree during maturation in hippocampal pyramidal neurons, although the general patterns are similar in both young and adult rats (Hertle and Yeckel 2007). Thus detailed studies on the distribution of InsP$_3$R in cortical pyramidal neurons and spatial profiles of InsP$_3$-dependent CICR with age are warranted.

Location-dependent cholinergic regulation of bAP-evoked dendritic Ca$^{2+}$ transients

CCh failed to enhance Ca$^{2+}$ transients evoked by single APs or by AP bursts in distal apical dendrites. The cholinergic effect on bAP-evoked Ca$^{2+}$ transients may require a minimum threshold [Ca$^{2+}$], with respect to the duration or amplitude of the Ca$^{2+}$ influx evoked by bAP to activate InsP$_3$-dependent CICR, as suggested by Nakamura et al. (2000). The correlation between the magnitude of the cholinergic effect and the amplitude of bAP-evoked Ca$^{2+}$ transients in proximal and middle dendrites (Figs. 1 and 2) supports this hypothesis. However, experimental evidence suggests that this scenario is unlikely in distal apical dendrites, where CCh treatment did not enhance the robust increase in [Ca$^{2+}$], that resulted from blocking A-type K$^+$ channels (Fig. 6). Furthermore, CCh did not modulate the supralinear Ca$^{2+}$ transients that were evoked by high-frequency APs (Fig. 7). Absence of CCh-induced increases in bAP-evoked Ca$^{2+}$ transients has also been reported in distal dendrites of BLA neurons, where uncaging of InsP$_3$ did not increase bAP-evoked Ca$^{2+}$ transients (Power and Sah 2007). Our results suggest that mAChRs and/or InsP$_3$-dependent CICR might be insufficient to evoke InsP$_3$-dependent CICR in distal apical dendrites. It appears that relatively little mAChR immunoreactivity is present in the distal apical dendrites of cortical layer 2/3 pyramidal neurons (Mrzljak et al. 1993; van der Zee and Luiten 1999). Because t-ACPD as well as CCh induced no increase in Ca$^{2+}$ transients in the present study, the downstream pathway from InsP$_3$ in these locations might be different from that in the soma, proximal apical, and basal dendrites (Hertle and Yeckel 2007; Power and Sah 2007). However, it is of interest whether other experimental conditions, such as synaptic stimulations, could induce Ca$^{2+}$ release from the stores in distal dendrites and spines.

Cholinergic regulation of bAP-evoked Ca$^{2+}$ transients in basal dendrites

Whereas only slight amplitude modulation of bAP has been detected in basal dendrites of layer 2/3 pyramidal neurons by the use of voltage-sensitive dyes (Antic 2003), direct patch-clamp recordings demonstrated much greater attenuation of bAP in basal dendrites than that in apical dendrites of layer 5 pyramidal neurons (Nevian et al. 2007). Layer 2/3 and layer 5 pyramidal neurons exhibited differences in dendritic propagation of somatic APs (Larkum et al. 2007). To date, direct electrophysiological recordings of the basal dendrite of layer 2/3 pyramidal neurons have not been reported. However, the greater amplitude of bAP-evoked Ca$^{2+}$ transients in distal basal dendrites observed in this experiment appears to result from the slight amplitude modulation of bAPs (Antic 2003), the increased duration of bAPs, and/or the surface-to-volume ratio (Nevian et al. 2007). In the present study, CCh uniformly increased Ca$^{2+}$ transients evoked by AP bursts along basal dendrites. However, secondary slow Ca$^{2+}$ transients were not observed in basal dendrites, except in the immediate vicinity of the soma (±10 µm of initial dendritic branches) in a few cells. As was observed in apical dendrites, cholinergic stimulation increased bAP-evoked Ca$^{2+}$ transients via mAChRs and InsP$_3$-dependent CICR. Interestingly, this cholinergic effect was observed when the Ca$^{2+}$ transients were evoked by AP bursts,
but not by single APs, which is similar to the pattern observed in middle apical dendrites. Although information on frequency-dependent modulation of the bAP amplitude in basal dendrites is equivocal (Antic 2003; Kampa and Stuart 2006), the results of the present study support that cholinergic stimulation reduces the frequency-dependent modulation of AP amplitude in basal dendrites as it does in apical dendrites (Tsubokawa and Ross 1997).

Because the depth from the cut surface of the slices to the measured areas did not differ between basal and distal apical dendrites under the experimental conditions used in the present study, differences in CCh diffusion between the two sites are unlikely. A similar result was observed with local application of CCh onto the dendritic tree (see Supplemental Fig. S1). Again, these findings suggest that the distribution of InsP$_3$-dependent CICR and/or mAChRs differs between basal and distal apical dendrites.

**Physiological implications of the location-dependent effect of cholinergic stimulation**

Propagating Ca$^{2+}$ waves in the soma and proximal dendrites control intrinsic excitability and the firing patterns in pyramidal neurons of the prefrontal cortex (Hagenston et al. 2008). Ca$^{2+}$ release evoked by muscarinic activation could control the neuronal excitability (Gulledge et al. 2007; Yamada et al. 2004). Dendritic Ca$^{2+}$ transients evoked by bAPs are heterogeneous in cortical pyramidal neurons due to the decreasing bAP amplitude, the complex geometry of the dendritic tree (Vetter et al. 2001), and the differential distribution of ion channels (Frick et al. 2003; Hoffman et al. 1997; Schiller et al. 1995; Smith et al. 2003) and of intracellular Ca$^{2+}$ stores (Blaustein and Golovina 2001; Johenning et al. 2002; Pozzo-Miller et al. 2000). These mechanisms allow pyramidal neurons to enhance their integration of synaptic activities via the use of multiple compartments (Antic 2003; Berger et al. 2003; Larkum et al. 2001; Poirazi et al. 2003). The complex spatial profile of the cholinergic effect on bAP-evoked dendritic Ca$^{2+}$ transients suggests an additional mechanism for the modulation of synaptic activity, which might be dependent on dendritic location (Froemke et al. 2005).

Because the presynaptic inputs to layer 2/3 pyramidal neurons in the primary sensory cortex appear to be segregated, terminating in localized dendritic areas of different cortical layers (Binzegger et al. 2004; Feldmeyer et al. 2002; Lukbe et al. 2003), modulation of synaptic activity by cholinergic stimulation might differ between distal apical and basal dendrites, where the most excitatory synaptic inputs terminate (Larkman 1991). Furthermore, differences between distal apical and basal dendrites in bAP-evoked Ca$^{2+}$ transient profiles and their cholinergic modulation may be involved in experience-dependent changes in altering the dynamics of cortical networks to allow learning of new information (Hasselmo and Bower 1992; Kimura et al. 1999).

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