Muscarinic Control of Dendritic Excitability and Ca\textsuperscript{2+} Signaling in CA1 Pyramidal Neurons in Rat Hippocampal Slice

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Egorov, Alexei V., Tengis Gloveli, and Wolfgang Müller. Muscarinic control of dendritic excitability and Ca\textsuperscript{2+} signaling in CA1 pyramidal neurons in rat hippocampal slice. J. Neurophysiol. 82: 1909–1915. 1999. The cholinergic system is critically involved in synaptic models of learning and memory by enhancing dendritic [Ca\textsuperscript{2+}] signals. Diffuse cholinergic innervation suggests subcellular modulation of membrane currents and Ca\textsuperscript{2+} signals. Here we use ion-selective microelectrodes to study spread of carbachol (CCh) after focal application into brain slice and subcellular muscarinic modulation of synaptic responses in CA1 pyramidal neurons. Proximal application of CCh rapidly blocked the somatic slow afterhyperpolarization (sAHP) following repetitive stimulation. In contrast, the time course of potentiation of the slow tetanic depolarization (STD) during synaptic input was slower and followed the time course of spread of CCh to the proximal dendritic segment. In dendritic recordings, CCh blocked a small sAHP, augmented the STD, and rather reduced dendritic action potentials. Augmentation of dendritic Ca\textsuperscript{2+} signals was highly correlated to augmentation of the STD. The NMDA receptor antagonist DL-2-amino-5-phosphonovaleric acid (APV) blocked ∼55% of the STD in control and during CCh application. In conclusion, muscarinic suppression of the proximal sAHP can augment firing and thereby Ca\textsuperscript{2+} responses. Dendritic augmentation of the STD by blockade of the sAHP and direct enhancement of N-methyl-D-aspartate (NMDA) receptor–mediated currents potentiates Ca\textsuperscript{2+} signals even when firing is not affected due to suprathreshold input. In this way, subcellular muscarinic modulation may contribute to parallel information processing and storage by dendritic synapses of CA1 pyramidial neurons.

METHODS
Preparation
Transverse hippocampal slices (300 μm thick) were prepared from adult ether-anesthetized Wistar rats (180–200 g) using a Campden manual vibratome slicer (Loughborough, England) and standard techniques (Misgeld et al. 1989; Müller and Misgeld 1986). The slices were continuously perfused with oxygenated (95% O\textsubscript{2}-5% CO\textsubscript{2}) artificial cerebrospinal fluid (ACSF) containing (in mM) 129 NaCl, 3 KCl, 1.6 CaCl\textsubscript{2}, 1.8 MgCl\textsubscript{2}, 21 NaHCO\textsubscript{3}, 1.25 NaH\textsubscript{2}PO\textsubscript{4}, and 10 glucose (37°C, pH 7.4).

Measurement of [CCh]o
For measurement of spread of the cholinergic agonist CCh, we used nominally K-selective microelectrodes (Corning Nr. 477317) back-filled with saline containing 100 mM CCh and 5 mM KCl. The resin was drawn into the presilanized ion-sensitive barrel of theta glass. With these high-impedance electrodes (2–50 GΩ), the sum of a Nernst-diffusion potential of ion activity and the extracellular dc-potential was monitored. Signals were fed into a differential amplifier (3 channels, 2 with input impedance >10\textsuperscript{14} Ω), which subtracts the dc-potential of the reference channel, filled with 154 mM NaCl, from the steady-state due to the difference in the standard potentials of Na\textsuperscript{+} and K\textsuperscript{+} caused by their different activities.
the ion-sensitive barrel signal. To suppress interference of disturbing ions in slices (choline), we performed all measurements and calibrations in the presence of 0.5 mM choline (Müller et al. 1988) and 10 μM atropine. CCh-sensitive electrodes were accepted as long as they responded with >14 mV for a CCh concentration change from 100 to 1,000 μM in the presence of 0.5 mM choline. Typical calibration responses are given in Fig. 1A. Using the water immersion objective as for the other experiments, the application micropipette and the CCh-sensitive electrode were both positioned in brain slice in the same depth below the upper surface, i.e., where cells were recorded to obtain good optical resolution (50–120 μm). CCh-induced rises in [K\(^+\)]\(_{o}\) were blocked by atropine (10 μM).

**Intracellular recording techniques**

Intracellular recordings were obtained using sharp microelectrodes pulled on a Brown-Flaming puller (Sutter Instruments, Novato, CA) from 1.2-mm borosilicate glass. Tips were filled with 5 mM Fura-2 (K\(^+\) salt, Molecular Probes, Eugene, OR) dissolved in 100 mM KCl, and electrodes were backfilled with 2 M K-acetate. Electrode resistance was initially 150–200 MΩ that dropped within 20 min to 70–120 MΩ when electrode solutions equilibrated. Intracellular recordings were made with a Neuro Data IR-283 current-clamp amplifier. After impalement of a neuron, Fura-2 was injected iontophoretically (0.5–1 nA, 5–15 min) until a signal over background of at least 3 was obtained. Neurons with resting potentials positive to −60 mV were rejected. Schaffer collaterals were stimulated over a width of up to 400 μm by bipolar glass-insulated silver electrodes. Electrophysiological data were recorded with an IBM-compatible PC, an ITC-16 AD interface (Instrutech, Elmont, NY), and WinTIDA data acquisition software (HEKA, Lambrecht/Pfalz, Germany). Eventually, the microelectrode was withdrawn to avoid mechanical problems and only Ca\(^{2+}\) responses were recorded.

**Ca\(^{2+}\) imaging**

Ratio imaging of Ca\(^{2+}\) was performed as described previously (Müller and Connor 1991a,b). In brief, cells were imaged with an upright microscope (Axioskop, Zeiss, Jena, Germany) and a long-distance water immersion objective (Achroplan 63×0.9). Digitized images were taken with a cooled charge-coupled device camera system (Photometrics, Tucson, AZ) with a Macintosh IIfx computer controlling image acquisition and display. Cytosolic-free Ca\(^{2+}\) concentrations were determined from background-corrected image pairs taken at 350- and 380-nm excitation using the ratio method (Gryniewicz et al. 1985).

**Drugs**

CCh (1–50 μM), atropine (1–10 μM, both from Sigma, Deisenhofen, Germany), and dl-2-amino-5-phosphonovaleric acid (APV, 30 μM, Tocris Cookson, Bristol, UK) were bath applied by continuous perfusion or by bolus application (Müller et al. 1988). Focal pressure pulse application of CCh (1–10 mM in ACSF, 1 bar, 10–50 ms) was performed through micropipettes with 2–3 μm tip diameter.

**Results**

Spread of CCh after focal application

We have shown previously that focal application of glutamate to apical dendrites of CA1 pyramidal neurons evokes spatially restricted increases of [Ca\(^{2+}\)]\(_{i}\) that are rapidly augmented by focal application of muscarinic agonists (Egorov and Müller 1999). Here we address subcellular muscarinic modulation of synaptic responses. Our hypothesis is that muscarinic modulation of the Ca\(^{2+}\)-dependent sAHP and of other currents locally augment excitability and Ca\(^{2+}\) responses while suppression of a strong proximal sAHP will globally affect the neuronal behavior. To correlate effects with the spatiotemporal pattern of activation of muscarinic receptors by CCh, we determined the concentration of CCh in the extracellular space and its dependence on the distance from the application site and on the time after application with CCh-sensitive microelectrodes. Figure 1 demonstrates calibration responses of the ion-selective electrode to CCh (A), the recording situation (B), and representative recordings of CCh concentration time courses at various distances from the application pipette (duration of pressure application 15–45 ms, C). During the initial 2–3 s a high CCh concentration of 10–50% of the pipette concentration is achieved in an area of ~120 μm diameter, depending on the duration of the pressure application of 15–45
responses to repetitive synaptic input (Schaffer collaterals 50 Hz, 1 s) in control and after focal application of CCh to the apical dendrite at ~280 μm from the soma. One–three seconds after CCh application, i.e., when CCh was able to activate muscarinic receptors only at a distal dendritic region of 60–120 μm in radius (cf. Fig. 1), the synaptic membrane potential response was not changed in the soma (B). Forty seconds after CCh application (C), i.e., when CCh has diffused to the soma (cf. inset), the sAHP following repetitive discharge is strongly reduced, and the slow depolarization during repetitive synaptic input is enhanced, whereas the decrease of amplitude of action potentials during the train is not affected. With weak train stimulation eliciting action potentials at ~50–70% of excitatory postsynaptic potentials (EPSPs) in control, CCh increased the number of action potentials evoked by this stimulus by 20–50%, depending on enhancement of the slow depolarization during the train and on the up to several minutes lasting depolarizing shift of the resting membrane potential.

Figure 3B demonstrates the Ca$$^{2+}$$ profile along the main apical dendrite before and during synaptic stimulation via Schaffer collaterals in another CA1 pyramidal cell. Peak Ca$$^{2+}$$ concentration is observed in the proximal part of the dendrite with moderate and steep gradients toward the distal dendrite and soma, respectively. Focal application of CCh to the apical dendrite at 350 μm from the soma augmented the intradendritic Ca$$^{2+}$$ response to synaptic input only after a delay of >10 s with a maximal effect at ~35 s (n = 16). This effect was most prominent at the proximal site of higher Ca$$^{2+}$$, and its time course was in agreement with the spread of CCh to this proximal site. This result might be due to a Ca$$^{2+}$$ dependence of the muscarinic modulation or due to a special importance of the proximal membrane. With distal synaptic Ca$$^{2+}$$ responses, we observed an effective augmentation again only after diffusion of CCh to the proximal membrane (including the entire apical dendritic tree, n = 5). Figure 4 demonstrates augmentation of a dendritic Ca$$^{2+}$$ signal at ~300 μm from the soma at 18 and 35 s after distal application of CCh (45 and 15 ms), exhibiting a strong correlation with the somatic/apical-proximal CCh concentration time course.
Dendritic electrophysiology

To directly address muscarinic modulation of dendritic membrane potential responses, we performed intradendritic recordings with sharp microelectrodes. Dendritic recordings showed that decay of action potential amplitudes during synaptic train input became more rapid and stronger with distance from the soma in addition to a decrease of action potential amplitude (Fig. 4Aa) (cf. Spruston et al. 1995). In our dendritic recordings, synaptic stimulation hardly evoked compound spiking or burst discharge (cf. Andreasen and Lambert 1995a,b). The mean of the sAHP following synaptically evoked repetitive discharge was significantly smaller in dendritic (200–300 μm) as compared with proximal recordings.

Bath application of CCh (1–50 μM) suppressed the dendritic sAHP elicited either by synaptic (n = 18) or by direct stimulation (n = 5, Fig. 5A and B). CCh dose-dependently enhanced the slow depolarization during synaptic train input across the whole membrane, i.e., in the dendrites (+75% with 20 μM CCh, n = 8) as well as in the soma (+73%, n = 5). Focal dendritic application of CCh (300 μm) started to suppress the dendritic sAHP at 280 μm from the soma within 1 s (n = 5) with a maximal effect after 20–30 s, whereas significant augmentation of the slow depolarization during synaptic train was slightly delayed (~10 s) with respect to blockade of the sAHP. The decay of dendritic action potential amplitudes during repetitive discharge was eventually somewhat reduced (Tsu-
bokawa and Ross 1997) but usually, concurrent to the degree of slow membrane depolarization during stimulation, rather enhanced by CCh (Fig. 5).

NMDA receptors contribute to augmentation of Ca$^{2+}$ signals

To study the importance of the enhancement of the STD during synaptic train input for the augmentation of intradendritic Ca$^{2+}$ signals, we combined intradendritic recording with Ca$^{2+}$ imaging ($n = 3$). Figure 6 demonstrates a strong correlation of the intradendritic Ca$^{2+}$ accumulation with the STD in control (A), at a low and high CCh concentration after bolus application into the bath perfusion (B and C) and recovery with wash out (D). Particular enhancement was observed for the STD and the Ca$^{2+}$ concentration during the second half of the train (peaks) versus the first half of the train (open symbol). Because muscarinic enhancement of NMDA receptor-mediated responses as well as muscarinic suppression of the Ca$^{2+}$-dependent sAHP can contribute to this muscarinic enhancement of the slow depolarization, we blocked the NMDA-mediated component by the receptor antagonist APV. Figure 7 shows muscarinic enhancement of the STD and suppression of the sAHP in control (A) and during superfusion with APV (30 μM, B). Blockade of NMDA receptors by APV suppressed the STD (+11.3 ± 2.3 mV at end of train, mean ± SE, n = 12, to 5.2 ± 0.8 mV, n = 3) (Collingridge et al. 1988; Herron et al. 1986) while not affecting the sAHP. Muscarinic activation increased this depolarization in APV to +9.7 ± 2.5 mV, as compared with +21 ± 6.4 mV in the absence of APV (20 μM CCh; because we observed no difference between soma and apical dendrite, data were pooled). The relative muscarinic augmentation of the STD was not affected by APV (+86% without APV versus +87% in APV).

DISCUSSION

Our results quantitatively characterize drug diffusion in the extracellular space after focal application of a drug that is not significantly degraded or taken up during the time span of interest. These results allow the determination of tissue regions with a drug concentration above certain levels of relevance. The correlation between augmentation of intradendritic Ca$^{2+}$ signals and the proximal CCh concentration after distal as well as after proximal CCh application indicates an important role for the proximal membrane in addition to integrative contributions from local muscarinic modulation of the dendritic tree.

Immunocytochemistry demonstrated a clustering of L-type Ca$^{2+}$ channels at the base of major dendrites in hippocampal pyramidal neurons (Westenbroek et al. 1990), and Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels has been found to effectively activate the sAHP (Marrion and Tavalin 1998). Synaptic stimulation evokes the strongest dendritic Ca$^{2+}$ signals at this site, most likely due to dendritic action potential. With synaptic stimulation missing threshold after initial firing due to activation of the sAHP, suppression of the sAHP is sufficient to increase firing and the intracellular Ca$^{2+}$ accumulation, like with firing evoked by direct stimulation or glutamate application. With strong suprathreshold synaptic train stimulation, the number of action potentials elicited is not changed by muscarinic activation. Then augmentation of the Ca$^{2+}$ signal is likely to depend on suppression of the sAHP resulting in stronger slow depolarization, relief of the Mg block of NMDA recep-

FIG. 6. Effects of bath application of CCh onto proximal (≈50 μm, see inset) dendritic membrane potential (top) and intradendritic Ca$^{2+}$ responses (bottom) to synaptic train stimulation (SC) recorded simultaneously. CCh bolus application (≈100 μM tissue concentration) results in reversible correlated augmentation of the slow depolarization during synaptic input and the intradendritic Ca$^{2+}$ response (A: control; B: 2 min; C: 4 min; D: 15 min after bolus application).

FIG. 7. Effects of blockade of NMDA receptors by DL-2-amino-5-phosphonovaleric acid (APV) onto synaptic membrane potential responses (soma) and their muscarinic modulation by bath-applied CCh. A and B: blockade of NMDA receptors selectively reduces the slow depolarization during synaptic input in control conditions as well as in the presence of CCh, but in the presence of APV CCh (−20 μM) still augments the slow depolarization. The sAHP following repetitive firing and its muscarinic blockade are not affected by APV (bottom traces).
tors, and stronger contribution of NMDA receptor–mediated Ca\(^{2+}\) influx. The increase of muscarinic effects during the initial 10 s after proximal application reflects presumably diffusion of CCh to and contribution of changes in dendrites up to \(\approx 150 \mu m\) from the soma. Because the effects work over 1 s once CCh bound to the receptors, it is unlikely that the time course is due to a slow cellular transduction process.

In addition to suppression of the sAHP, muscarinic activation can enhance synaptic Ca\(^{2+}\) signals by direct augmentation of NMDA receptor–mediated currents and of amplitude and duration of action potentials. Our results with trains of suprathreshold EPSPs demonstrate that the slow depolarization during repetitive synaptic input is largely due to current flow through NMDA receptor channels (Fig. 7). This slow depolarization effectively contributes to dendritic Ca\(^{2+}\) signals and their potentiation (Fig. 6), even in the absence of action potentials when evoked by focal glutamate application (Egorov and Müller 1999). Augmentation of this slow depolarization requires higher CCh concentrations and has been shown, in the case of glutamate application, to be lithium sensitive. Both points support mediation by M1 receptors as opposed to M2 receptors in suppression of the sAHP (Markram and Segal 1992; Müller and Misgeld 1986).

We have shown previously that suppression of the sAHP is sufficient to strongly augment Ca\(^{2+}\) signals evoked by direct depolarization due to increased firing (Müller and Connor 1991a). Synaptically as well as antidromically evoked distal dendritic action potentials have been demonstrated to be enhanced in amplitude by CCh (Hoffman and Johnston 1999; Tsubokawa and Ross 1997). We have observed eventually slight increases in spike amplitude, but often no change or a decrease. While we elicited action potentials directly or orthodromically using a wide stimulation electrode and used sharp microelectrode versus perforated and whole cell patch-clamp recording, these differences are not likely to explain the discrepancy. The decrease of action potential amplitudes is presumably due to suppression of the sAHP and augmentation of the synthetically evoked slow membrane depolarization, causing inactivation of Na\(^{+}\) channels. In addition, Cantrell et al. and others have shown that high concentrations of CCh (\(\geq 5 \mu M\)) inhibit the sodium current through activation of PKC with a \(K_I\) of \(\approx 8 \mu M\) (Cantrell et al. 1996; Mittmann and Alzheimer 1998). With focal as well as bolus applications used in this study, we obtained CCh concentrations in this range (10–50 \(\mu M\)) and above, in contrast to CCh concentrations enhancing dendritic action potentials (Hoffman and Johnston 1999; Tsubokawa and Ross 1997). In addition, there are methodological differences that might stabilize the dendritic balance between inward and outward currents and hence dendritic action potentials; e.g., Tsubokawa and Ross as well as Hoffman and Johnston reduced fast GABAergic inhibition using an antagonist, age of animals, strain, and temperature.

In summary, our results demonstrate that muscarinic input modulates membrane excitability and synaptic Ca\(^{2+}\) signals subcellularly with an important proximal and a distributed dendritic component. The dendritic component clearly involves 1) suppression of the sAHP, allowing increased firing and depolarization contributing to removal of the Mg\(^{2+}\) block of NMDA channels; 2) direct augmentation of NMDA receptor–mediated currents (Markram and Segal 1992); and 3) inhibition of A-type K channels (Nakajima et al. 1986). Local augmentation of distal dendritic responses presumably has been below the threshold of detection due to widespread synaptic activation, electrotonic coupling, and limited temporal and spatial resolution of the methods used. The dendritic component became evident in somatic recordings only when affecting a significant fraction (\(\approx 150 \mu m\) in radius) of the dendritic tree. The proximal membrane has a particular importance not only for the somatic but also for the dendritic membrane potential and Ca\(^{2+}\) signals, most likely due to clustering of Ca channels linked to activation of a strong sAHP. The somatic membrane potential is of particular importance for the input-output function of the cell including backpropagation of action potentials into the dendritic tree. Inhibition of Na\(^{+}\) and Ca\(^{2+}\) channels at higher agonist concentration (Cantrell et al. 1996; Toselli and Lux 1989) will limit intradendritic Ca\(^{2+}\) levels during strong excitation. In this way, muscarinic activation will facilitate induction of Ca\(^{2+}\)-dependent synaptic plasticity while avoiding deleterious Ca\(^{2+}\) overload. Whether in vivo the diffuse cholinergic input independently modulates proximal and distal membrane regions remains to be investigated.

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