Muscarnic Enhancement of R-Type Calcium Currents in Hippocampal CA1 Pyramidal Neurons

Chao Tai,1,2* J. Brent Kuzmiski,1,2* and Brian A. MacVicar1
1Brain Research Center, Department of Psychiatry, University of British Columbia, Vancouver, British Columbia V6T 2B5, Canada, and 2Hotchkiss Brain Institute, Calgary, Alberta T2N 4N1, Canada

The “toxin-resistant” R-type Ca2+ channels are expressed widely in the CNS and distributed mainly in apical dendrites and spines. They play important roles in regulating signal transduction and intrinsic properties of neurons, but the modulation of these channels in the mammalian CNS has not been studied. In this study we used whole-cell patch-clamp recordings and found that muscarinic activation enhances R-type, but does not affect T-type, Ca2+ currents in hippocampal CA1 pyramidal neurons after N, P/Q, and L-type Ca2+ currents selectively were blocked. M1/M3 cholinergic receptors mediated the muscarinic stimulation of R-type Ca2+ channels. The signaling pathway underlying the R-type enhancement was independent of intracellular [Ca2+] changes and required the activation of a Ca2+-independent PKC pathway. Furthermore, we found that the enhancement of R-type Ca2+ currents resulted in the de novo appearance of Ca2+ spikes and in remarkable changes in the firing pattern of R-type Ca2+ spikes, which could fire repetitively in the theta frequency. Therefore, muscarinic enhancement of R-type Ca2+ channels could play an important role in modifying the dendritic response to synaptic inputs and in the intrinsic resonance properties of neurons.

Key words: acetylcholine; muscarinic; R-type calcium spikes; α1E calcium subunits; CaV2.3; T-type calcium currents; theta; epilepsy; oscillations; hippocampus

Introduction

R-type voltage-sensitive Ca2+ currents originally were identified as the high voltage-activated (HVA) Ca2+ currents that are resistant to the antagonists ω-conotoxin MVIIC, ω-conotoxin-GVIA, ω-agatoxin IVA, and the dihydropyridines (Zhang et al., 1993; Randall and Tsien, 1995). In hippocampal CA1 pyramidal neurons the R-type voltage-sensitive Ca2+ channels (VSCCs) are highly expressed in distal dendrites (Christie et al., 1995; Magee and Johnston, 1995) and are thought to be primarily responsible for Ca2+ influx in dendrites and spines (Sabatini and Svoboda, 2000; Yasuda et al., 2003). R-type Ca2+ currents are involved in generating action potential bursts and afterdepolarizations (Magee and Carruth, 1999; Metz et al., 2005) and in the induction of synaptic plasticity (Isomura et al., 2002; Breustedt et al., 2003; Dietrich et al., 2003; Yasuda et al., 2003).

Modulation of R-type Ca2+ currents could have profound impacts on dendritic excitability via modification of intrinsic firing patterns and the integrative properties of dendrites. Brief trains of backpropagating action potentials have been shown to depress Ca2+ entry through R-type VSCCs located in dendritic spines and thereby block theta burst-induced long-term potentiation (LTP) (Yasuda et al., 2003). In expression systems the R-type Ca2+ currents caused by recombinant CaV2.3 (α1E) VSCCs (Piedras-Renteria and Tsien, 1998; Sochivko et al., 2002; Bannister et al., 2004) are stimulated by the activation of coexpressed muscarinic (Meza et al., 1999; Melliti et al., 2000; Bannister et al., 2004) or metabotropic glutamate receptors (mGlurRs) (Stea et al., 1995). However, the modulation of R-type Ca2+ currents by muscarinic or metabotropic receptors has not been examined in native neurons in brain slices or in vivo. Therefore, we used whole-cell recordings to examine whether Ca2+ currents caused by R-type VSCCs are enhanced in hippocampal brain slices by muscarinic activation. We show that both R-type Ca2+ currents and spikes are enhanced by the stimulation of muscarinic receptors in CA1 pyramidal neurons. This is in striking contrast to the extensively studied depression of N-, P/Q-, and L-type Ca2+ currents by the activation of muscarinic receptors (Gahwiler and Brown, 1987; Shapiro et al., 1999, 2001; Stewart et al., 1999). Furthermore, initiation of dendritic Ca2+ spikes has been suggested to play a role in generating or shaping neuronal network oscillations (Kamondi et al., 1998; Buzsaki, 2002). Interestingly, we found that muscarinic stimulation leads to remarkable and novel changes in the R-type Ca2+ spike firing pattern. After muscarinic receptor stimulation the enhanced R-type Ca2+ spikes repetitively fired at theta frequencies (6–10 Hz), and blocking R-type VSCCs depressed carbachol-induced spontaneous field potential theta oscillations, suggesting that enhanced R-type calcium spikes play a role in dendritic bursting and network oscillations. Therefore, muscarinic receptor activation in hippocampal neurons will alter profoundly the dendritic integrative and intrinsic resonance properties by shifting the normal...
pattern of Ca\(^{2+}\) entry from the slowly inactivating N-, P/Q-, and L-type VSCCs to domination by the HVA rapidly inactivating R-type VSCCs.

Materials and Methods

Hippocampal slice preparation. Hippocampal slices were prepared from Sprague Dawley rats, aged postnatal days 13–16, according to standard procedures (Fraser and MacVicar, 1996). Our experiments were approved by the Canadian Council for Animal Care and the University of British Columbia Animal Care Committee. All experiments were conducted in strict accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals. Briefly, the rats were anesthetized deeply with halothane and decapitated rapidly. The brain was removed quickly, and horizontal hippocampal slices (~400 μm) were cut with a vibratome (VT1000, Leica, Willowdale, Ontario, Canada) in chilled (0–4°C) slicing containing the following (in mM): 75 sucrose, 87 NaCl, 25 NaHCO\(_3\), 25 d-glucose, 2.5 KCl, 1.25 NaH\(_2\)PO\(_4\), 0.5 CaCl\(_2\), and 7.0 MgCl\(_2\), pH 7.3. Then the slices were transferred to a storage chamber containing fresh artificial CSF (ACSF) (2 ml/min). Patch electrodes (3–5 MΩ) were verified by the recording of evoked field EPSPs. Glass micropipettes filled with ACSF were used to stimulate electrically the Schaffer collateral pathway (0.03 Hz). Stable recordings (~20 min) with a population spike that had peak-to-peak amplitudes of >10 mV were used.

Reagents. TTX, ω-conotoxin-GVIA, and ω-agatoxin IVA were purchased from Alamone Labs (Jerusalem, Israel); ω-conotoxin MVIIIC from Bachem (Torrance, CA); nickel (II) chloride from J.T. Baker (Paris, KY); pirenzepine, protein phosphatase 2 (PP2), and 2-[1-(3-dimethylaminopropyl)[indol-3-yl]-3-(indol-3-yl) maleimide (GO-19203x) from Tocris (Ellisville, MO); BAPTA from Molecular Probes (Eugene, OR); 3-[1-3-(aminodinothio)-propyl]-H-indol-3-yl-3-(1-methyl-H-indol-3-yl)maleimide (Ro 31-8220), 2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl-N-methylbenzylamine) (KN-93), and 12-(2-cya-noethoxy)-6,7,12,13-tetrahydro-13-methyl-5-oxo-2,3-(2,3-a)pyrrololo(3,4-c)-carbazole (Go 6976) from Calbiochem (La Jolla, CA); all other reagents were purchased from Sigma (St. Louis, MO).

Data analysis. Data were analyzed with Clampfit 9.0 (Molecular Devices). In all cases Student’s t tests were used for statistical comparisons, with p < 0.001 considered significant. Values are reported as the mean ± SEM.

Results

Carbachol enhances toxin-resistant HVA R-type currents but does not affect low voltage-activated T-type Ca\(^{2+}\) currents

We tested the hypothesis that muscarinic receptor stimulation enhances R-type VSCCs in CA1 pyramidal neurons similar to the muscarinic enhancement of recombinant Ca\(_{\alpha,2.3}\) VSCCs described in human embryonic kidney (HEK) 293 cells (Melliti et al., 2000; Bannister et al., 2004). We performed voltage-clamp recordings to characterize the cholinergic modulation of the toxin-resistant Ca\(^{2+}\) currents, which were isolated with a mixture containing TTX, nifedipine, and VSCC toxins (as described in Materials and Methods). CA1 pyramidal neurons from hippocampal slices were patch clamped with a Cs\(^{+}\)- and TEA\(^{-}\)-based intracellular solution to suppress K\(^{+}\) channels and to minimize muscarinic-mediated effects on K\(^{+}\) channels. The ACSF contained Cs\(^{+}\) and 4-AP to block potassium current and to increase the space-clamp efficiency. Under these conditions we observed an enhancement of HVA Ca\(^{2+}\) currents. In the presence of the VSCC blocker mixture toxin-resistant low (T-type) and high (R-type) voltage-activated transient Ca\(^{2+}\) currents were recorded in all cells (Fig. 1A). The classification of currents was based on the potentials for activation, toxin resistance, and Ni\(^{2+}\) sensitivity. Only the HVA R-type current was enhanced dramatically after the application of carbachol (n = 13) (Fig. 1B). This carbachol-mediated stimulation was reversible (n = 6) (Fig. 1C), and the currents were sensitive to 50 μM Ni\(^{2+}\) (n = 5) (Fig. 1D). Strikingly, the I–V curve of the Ca\(^{2+}\) currents showed that the T-type component (observed as a shoulder in the I–V at –60 to –30 mV) was not affected, whereas the peak of the R-type current was enhanced dramatically in carbachol (Fig. 1E). The averaged I–V curve before and after carbachol treatment is shown in Figure 2A (n = 7). The peak amplitudes of T-type and R-type currents are plotted in Figure 2B, showing that the T-type component did not change (99.6 ± 2% of control; p = 0.76; n = 10), whereas the R-type component increased significantly (154 ± 4% of control; p < 0.0001; n = 13). The example traces for T-type and R-type are shown in Figure 2C. The enhanced amplitude of the R-type Ca\(^{2+}\) current was associated consistently with a significant shift of the voltage-dependent activation curve to negative potentials, with a change in the voltage for half-maximal activation (Vhalfact) of ΔVact = −4.2 ± 0.7 mV; p < 0.001; n = 7) (Fig. 2D). The enhancement of R-type Ca\(^{2+}\) currents was reversible and blocked by atropine (1 μM), demonstrating that it was attributable to muscarinic receptor activation by carbachol (n = 6) (Fig. 2E). Poole data for
muscarinic modulation of R-type and T-type VSCCs are shown in Figure 2 F (for each group, n = 5). These results suggest that, in CA1 pyramidal neurons, muscarinic activation enhances only the HVA R-type Ca\(^{2+}\) currents, but not the low voltage-activated (LVA) T-type Ca\(^{2+}\) currents.

To verify additionally that T-type Ca\(^{2+}\) currents were not modulated by muscarinic activation, we first blocked all HVA Ca\(^{2+}\) currents by perfusing 30 μM Cd\(^{2+}\) instead of the strategy used above with toxins and nifedipine. This is effective for isolating T-type Ca\(^{2+}\) currents, because the HVA Ca\(^{2+}\) currents are much more sensitive to low concentrations of Cd\(^{2+}\) (Ozawa et al., 1989; Mogul and Fox, 1991; Avery and Johnston, 1996; Guenard, 1996). Under these conditions a LVA component was observed with no apparent HVA component (n = 5; p < 0.0001; n = 7) (Fig. 3 E). These results suggest the existence of multiple subunits of T-type VSCCs in CA1 pyramidal neurons, which is consistent with previous in situ hybridization work (Talley et al., 1999). These results

**Figure 1.** Carbachol enhances toxin-resistant HVA Ca\(^{2+}\) currents. Whole-cell patch-clamp recordings were performed with a Cs\(^{+}\)- and TEA\(^{-}\)-based intracellular solution. Slices were preincubated with toxins (as described in Materials and Methods) and perfused with TTX (1.2 μM) and nifedipine (20 μM). A, In voltage-clamp mode, a well clamped T-type current was recorded. B, The current was enhanced by the application of carbachol (30 μM). This enhancement was reversible (C) and sensitive to Ni\(^{2+}\) (30 μM; D). E, I–V relations of this cell before and after treatment, showing that the T-type component (observed as a shoulder at –60 to –30 mV in the I–V) was not affected, whereas the peak of the R-type current was enhanced dramatically in carbachol (CCH).

**Figure 2.** Muscarinic activation enhances R-type, but not T-type, Ca\(^{2+}\) current. A–C, HVA, but not LVA, component is stimulated by muscarinic activation. The mean I–V relationships before and after carbachol (CCH) treatment from seven cells are shown in A. The peak current amplitudes and sample traces are shown in B. C (1, Control; 2, CCH). D, The Ca\(^{2+}\) channel activation curve analyzed from peak currents and estimated conductance is shifted significantly to the left. The result was similar when tail currents were analyzed (data not shown). The result was fit with a Boltzmann equation. E, Time course of the peak HVA Ca\(^{2+}\) current shows that carbachol (CCH) stimulation of R-type VSCCs is reversible and is blocked by the muscarinic receptor antagonist atropine (1 μM). F, Mean data for the muscarinic modulation of the LVA and HVA Ca\(^{2+}\) current components. All recordings were obtained in TTX and VSCC blockers (as described in Materials and Methods). Error bars indicate the mean ± SEM. Asterisk indicates significant change (*p < 0.001).
confirmed that, in CA1 pyramidal neurons, muscarinic activation had no effect on T-type Ca\(^{2+}\) currents.

M\(_1\)-/M\(_3\) cholinergic receptors mediate muscarinic stimulation of R-type VSCCs

The ability of atropine (1 \(\mu\)M) to block the stimulation of carbachol in R-type Ca\(^{2+}\) currents demonstrates that muscarinic receptors mediate this enhancement. In the hippocampus four of the muscarinic subtypes (M\(_1\)–M\(_4\)) are expressed abundantly (Vilaro et al., 1993; Levey et al., 1995; Rouse et al., 1999). To determine further the muscarinic receptor subtypes involved in the enhancement of R-type Ca\(^{2+}\) currents, we tested the antagonists and agonists for M\(_1\)–M\(_4\) cholinergic receptors. We found that both pirenzepine (1 \(\mu\)M; \(n = 5\)), a M\(_1\)-specific antagonist, and 4-diphenylacetoxy-N-methylpiperidine (4-DAMP; 1 \(\mu\)M; \(n = 4\)), an antagonist with equal affinity to both M\(_1\) and M\(_3\) receptors, could reverse the carbachol enhancement of R-type Ca\(^{2+}\) currents (Fig. 4A–C). In contrast, the M\(_2\)/M\(_4\) antagonists methoctramine and tropicamide (1 \(\mu\)M; M\(_2\) and M\(_4\) antagonists) on the enhancement of R-type VSCCs. D–F, M\(_1\)-agonist MCN-A-343 (100 \(\mu\)M) mimicked and occluded the stimulation of R-type VSCCs by carbachol (CCH). I–V curves and sample traces are shown in D, E (1, Control; 2, MCN-A-343; 3, CCH). F, Mean data for the effects of McN-A-343 and the subsequent application of carbachol (CCH) on R-type VSCCs. All recordings were obtained in TTX and VSCC blockers (as described in Materials and Methods). Error bars indicate the mean \pm SEM. Asterisk indicates significant change (* \(p < 0.001\)).
the muscarinic enhancement of R-type Ca\(^{2+}\) currents is mediated by M\(_1\)/M\(_3\) subtypes.

**Muscarinic modulation of R-type VSCCs requires a Ca\(^{2+}\)-independent PKC pathway**

In recombinant systems the stimulation of Ca\(_{\text{V}2.3}\) Ca\(^{2+}\) currents is dependent on phosphorylation that is mediated by a pathway coupled to a pertussis toxin-insensitive G\(_q\) subunit (G\(_{\alpha_q}\)) (Bannister et al., 2004). M\(_1\)/M\(_3\) receptors couple to G\(_q\) subunits to stimulate phospholipase C\(_{\beta}\) (PLC\(_{\beta}\)), which initiates phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) turnover. This leads to the production of diacylglycerol (DAG) and IP\(_3\)-mediated Ca\(^{2+}\) release, which in turn activates protein kinase C (PKC). Basically, there are three groups of PKCs: the Ca\(^{2+}\)- and DAG-dependent isoforms (group I), the Ca\(^{2+}\)-independent but DAG-dependent isoforms (group II), and the atypical isoforms (group III). We examined the signaling mechanisms underlying the enhancement of R-type Ca\(^{2+}\) currents by first testing the Ca\(^{2+}\) dependence and then by examining the sensitivity to different PKC inhibitors. A high concentration of high-affinity Ca\(^{2+}\) chelator BAPTA (10 mM) was used in the pipette solution, which has proved to be sufficient to block completely the carbamol-induced plateau potentials or tail currents in our lab (Fraser and MacVicar, 1996; Kuzmiski and MacVicar, 2001). We found that 10 mM BAPTA could not prevent the carbamol enhancement of R-type VSCCs (Fig. 5A, D), suggesting it involves a Ca\(^{2+}\)-independent pathway. To investigate the potential involvement of PKC in the enhancement of R-type VSCCs, we preincubated slices for >30 min with the broad spectrum PKC antagonists GF 109203x (10 \(\mu\)M; \(n = 5\)) or Ro 31-8220 (10 \(\mu\)M; \(n = 5\)). In the presence of these PKC antagonists carbamol did not enhance R-type Ca\(^{2+}\) currents (Fig. 5B, E), suggesting that PKCs are involved in this modulation. Interestingly, in the presence of PKC inhibitors (GF 109203x or Ro 8332) a significant suppression of R-type Ca\(^{2+}\) currents was observed by muscarinic stimulation (Fig. 5B, E). This inhibitory effect probably results from the activation of pertussis toxin-sensitive G-protein-coupled M\(_2\)/M\(_4\) receptors and G\(_q\) subunit-mediated inhibition (Meza et al., 1999; Bannister et al., 2004). Because we observed a stable enhancement of R-type currents in normal conditions, we did not study this inhibitory effect further. To determine which group of PKCs is involved, we applied the specific group I (Ca\(^{2+}\)-dependent) PKC inhibitor Go 6976 (10 \(\mu\)M; \(n = 5\)). Application of Go 6976 did not block the carbamol-mediated enhancement of R-type Ca\(^{2+}\) currents (Fig. 5C, E). Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and Src-family signaling pathways also have been reported to be involved in some muscarinic-activated pathways. However, we found that inhibitors of CaMKII (KN-93, 10 \(\mu\)M; \(n = 6\)) and Src-kinase (PP2, 10 \(\mu\)M; \(n = 5\)) had no effect on the muscarinic stimulation of R-type Ca\(^{2+}\) currents at concentrations that have been reported to be effective in brain slices (Fig. 5E) (Zhao et al., 2004; Grishin et al., 2005; Huang et al., 2005). This pattern of sensitivity to PKC antagonists and insensitivity to BAPTA suggests the involvement of Ca\(^{2+}\)-independent PKCs in the muscarinic effect on R-type Ca\(^{2+}\) currents.

**Muscarinic enhancement of R-type Ca\(^{2+}\) spikes**

Current-clamp recordings also were performed to characterize the cholinergic modulation of the toxin-resistant Ca\(^{2+}\) spikes. CA1 pyramidal neurons from hippocampal slices also were patch-clamped with a Cs\(^{+}\)-based intracellular solution to suppress K\(^{+}\) channels and to minimize muscarinic-mediated effects on K\(^{+}\) channels. When recordings were obtained from CA1 neu-

![Figure 5](image-url).
rons in TTX without a blocking of any Ca\(^{2+}\) currents, large-amplitude prolonged plateau Ca\(^{2+}\) spikes were evoked by injection of brief (40 ms) intracellular current pulses (n = 7) (Fig. 6A,B). Carbachol depressed the plateau Ca\(^{2+}\) spikes, consistent with a muscarinic depression of L-, N-, and P/Q-type VSCCs, leaving only a transient HVA Ca\(^{2+}\) spike. A significant portion of this calcium spike in carbachol could be attributable to R-type Ca\(^{2+}\) currents, because it was Ni\(^{2+}\)-sensitive (n = 4). These results suggest that muscarinic receptor activation in hippocampal neurons could shift the normal pattern of Ca\(^{2+}\) entry from the slowly inactivating N-, P/Q-, and L-type VSCCs to domination by the HVA rapidly inactivating R-type VSCCs.

To delineate rigorously the direct action of carbachol on the toxin-resistant Ca\(^{2+}\) spikes, we isolated them with a mixture containing TTX, nifedipine, and toxins, as shown in previous voltage-clamp experiments. Under these conditions depolarization of neurons with intracellular current injection often led to the generation of a transient HVA Ca\(^{2+}\) spike (n = 50 of 69). The Ca\(^{2+}\) spike inactivated during the depolarizing command pulses, consistent with a Ca\(^{2+}\) spike mediated by rapidly inactivating HVA R-type Ca\(^{2+}\) currents (Randall and Tsien, 1997). However, even with strong current injection (≥0.5 nA) numerous cells (28%; n = 19 of 69) remained silent. As shown previously with current-clamp recordings (Kuzmisky et al., 2005), subsequent bath application of carbachol resulted in persistent enhancement of R-type Ca\(^{2+}\) spiking in both the spiking and initially silent neurons (Fig. 6C,D). The initially silent neurons exhibited transient Ca\(^{2+}\) spikes in the presence of carbachol (n = 19) (Fig. 6C,D). In neurons that initially showed an R-type Ca\(^{2+}\) spike, carbachol induced an increase in spike amplitude (ΔAP\(_{\text{AMP}}\) = 5.7 ± 0.7 mV; p < 0.0001; n = 50), a decrease in the threshold for spike activation (ΔAPT\(_{\text{TH}}\) = −6.7 ± 0.8 mV; p < 0.0001; n = 50), and a reduction in the current injection required to evoke a spike (ΔI\(_{\text{TH}}\) = −80 ± 12 pA; p < 0.0001; n = 50) (Fig. 6F). These enhanced spikes were blocked by the application of Ni\(^{2+}\) (50 μM; n = 20) (Fig. 6E,G,H), which at this concentration is selective for R- and T-type VSCCs. This enhancement was not caused by a muscarinic-mediated increase in input resistance (R\(_{\text{IN}}\)) (control R\(_{\text{IN}}\) = 115.7 ± 5.9 MΩ vs carbachol R\(_{\text{IN}}\) = 118.0 ± 6.1 MΩ; p = 0.77; n = 69). The application of high concentrations of nimodipine (20 μM) sufficient to block partially the T-type VSCCs had no effect on the carbachol-enhanced HVA Ca\(^{2+}\) spikes (percentage of ΔAP\(_{\text{AMP}}\), 3.5 ± 2.6%; p = 0.25; percentage of ΔdV\(_{\text{m}}\)/dt\(_{\text{max}}\), −5.2 ± 9.1%; p = 0.38; n = 5) (Fig. 6G,H). In addition, the Ca\(^{2+}\) spikes were only weakly voltage dependent when held at depolarized voltages (from −70 to −60 mV) (percentage of ΔAP\(_{\text{AMP}}\), 2.9 ± 4.8%; p = 0.82; percentage of ΔdV\(_{\text{m}}\)/dt\(_{\text{max}}\) = −14.6 ± 10.6%; p = 0.24; n = 5) (Fig. 6G,H). Consistent with our voltage-clamp results, we concluded that R-type, but not T-type, Ca\(^{2+}\) currents generated the toxin-resistant Ca\(^{2+}\) spikes.

**Muscarinic enhancement of R-type Ca\(^{2+}\) spikes contributes to carbachol-induced theta burst oscillations**

The cholinergic system is implicated in the generation of theta both in vitro and in vivo (Buzsaki, 2002). During theta in vivo the HVA Ca\(^{2+}\) spikes oscillate rhythmically at theta frequencies in the dendrites of CA1 pyramidal neurons and may contribute to current generation and amplification of theta (Kamondi et al., 1998; Buzsaki, 2002). However, the VSCC subtypes that contribute to dendritic oscillations during theta are unknown. We examined the possibility that muscarinic-enhanced R-type Ca\(^{2+}\) spikes can resonate at theta frequency and thereby contribute to the generation of dendritic oscillations. To evaluate the firing
occurred at regular intervals of 101.4 ± 0.6 Hz (Fig. 8A). Field potential recordings showed a peak power spectra of 0.016 ± 0.0071 mV²/Hz at a mean frequency of 8.3 ± 0.6 Hz (n = 8) (Fig. 8B). The spontaneous oscillatory bursts occurred at regular intervals of 101.4 ± 10.9 s (n = 8) and lasted for 21.4 ± 2.6 s. Theta burst oscillations closely resembled those previously described (Bland et al., 1988). Subsequent application of a low concentration of Ni²⁺ depressed the oscillatory activity, as shown by an almost complete abolition of the power in the theta frequency range (n = 8 of 8) (Fig. 8A,B). In five of eight slices the oscillatory bursts were abolished completely. In the other three slices Ni²⁺ reduced the amplitude of the theta burst oscillations and increased the interburst interval without signifi-
cant effects on burst durations (19.3 ± 2.8 s; n = 3) or the frequency of the oscillations (8.3 ± 0.4 Hz). Disruption of the theta bursts often was accompanied by a change in the field events to a slower, more interictal-like pattern. Because we demonstrated that T-type VSCCs are not affected by cholinergic stimulation, these results suggest that Ni²⁺ suppressed the carbachol-induced spontaneous field potential theta oscillations by depressing R-type VSCCs. Therefore, our results indicate that in CA1 pyramidal neurons the enhancement of R-type VSCCs could contribute to muscarinic-induced theta frequency oscillations.

Discussion

Our results show that muscarinic activation stimulates R-type Ca²⁺ currents in hippocampal CA1 neurons that can lead to de novo activation of R-type dependent Ca²⁺ spikes. This enhancement likely is mediated via a Ca²⁺-independent PKC pathway because it is blocked by broad spectrum inhibitors to PKC, but not by an inhibitor of Ca²⁺-dependent PKC, and it is insensitive to chelation of intracellular Ca²⁺ by BAPTA. In contrast to the enhancement of the R-type currents, T-type Ca²⁺ currents were not modulated by muscarinic receptor activation. The muscarinic-mediated enhancement of transient R-type Ca²⁺ spikes resulted in remarkable changes in the firing pattern of R-type Ca²⁺ spikes and could contribute to theta oscillations.

Mechanisms underlying muscarinic enhancement of R-type VSCCs

R-type Ca²⁺ currents in hippocampal neurons are attributable mainly to Caᵥ₂.₃ subunits (Schövko et al., 2002, 2003), although there is still some controversy as to the proportion of R-type Caᵥ₂.₃ currents that remains in the Caᵥ₂.₃ knock-out mouse (Wilson et al., 2000). In recombinant systems Caᵥ₂.₃ subunit Ca²⁺ currents are enhanced by Goq₁₁-coupled muscarinic receptor activation (Bannister et al., 2004; Kamatchi et al., 2004). Here we show a similar enhancement of R-type Ca²⁺ currents in hippocampal neurons also mediated by muscarinic M₁/M₄ receptor stimulation. Hippocampal pyramidal neurons express high levels of postsynaptic M₁ and M₃ receptors (Vilario et al., 1993; Levey et al., 1995; Rouse et al., 1999). These receptors are Goq₁₁ coupled, and their activation results in the generation of DAG and IP₃, via PLC activation. DAG and IP₃, in turn activate PKC and IP₃ receptor pathways, respectively. Similar to previous work in HEK cells and Xenopus oocytes (Bannister et al., 2004; Kamatchi et al., 2004), the muscarinic stimulation of R-type VSCCs in hippocampal neurons was independent of intracellular Ca²⁺ and required the activation of Ca²⁺-independent group II PKCs. All three groups of PKCs are expressed in rat hippocampus (Naik et al., 2000), and PKCθ from among the group II PKCs (δ, ε, η, and θ) may be the isoform of PKC that is involved. PKCθ is highly expressed in rat hippocampal CA1 pyramidal neurons (McNamara et al., 1999; Tang et al., 2004), and in response to muscarinic stimulation PKCθ is activated and translocated to plasma membrane (Brown et al., 2005). In recombinant systems the co-expression of a dominant-negative PKCθ blocked the muscarinic stimulation of Caᵥ₂.₃ subunits, whereas the blocker of PKCε could not (Bannister et al., 2004). However, we could not rule out definitively the contribution of other isoforms. In PKC inhibitors we observed a suppression of R-type Ca²⁺ currents by muscarinic stimulation, suggesting other inhibitory pathways. This inhibition may result from the activation of pertussis toxin-sensitive G-protein-coupled M₁/M₄ receptors and Gβγ subunit-mediated inhibition (Meza et al., 1999; Bannister et al., 2004).
R-type versus other VSCC types
Muscarinic enhancement of R-type VSCCs is strikingly opposite to muscarinic depression of all other HVA VSCCs (N-type, P/Q-type, and L-type) (Gahwiler and Brown, 1987; Shapiro et al., 1999, 2001; Stewart et al., 1999). Christie and colleagues (1995) showed that in the soma and basal dendrites (≥50 μm) all of the HVA VSCCs as well as LVA VSCCs contribute to spike-triggered Ca^{2+} entry. In contrast, T-type and Ni^{2+}-sensitive R-type VSCCs predominantly underlie spike-triggered Ca^{2+} entry in apical dendrites (≥100 μm) (Christie et al., 1995). Single channel analysis also shows that all VSCC types are expressed in the soma, whereas in dendrites T- and R-type VSCCs predominate (Magee and Johnston, 1995). Action potential or depolarization-induced Ca^{2+} influx in dendrites and spines also has been shown to be mediated mainly by R-type VSCCs (Sabatini and Svoboda, 2000; Yasuda et al., 2003). The distinct distribution in apical dendrites and the unique modulation of R-type VSCCs suggest that they play a different role and underlie distinct cellular functions from other types of VSCCs, such as synaptic integration and plasticity.

Despite the general suppression of Ca^{2+} currents, muscarinic activation paradoxically increases intracellular Ca^{2+} accumulations in the dendrites and spines from depolarization or synaptic stimulation (Muller and Connor, 1991, 1992; Tsunokawa and Ross, 1997; Beier and Barish, 2000). K⁺ conductance blockade and the IP₃-mediated release of Ca^{2+} from intracellular stores are thought to contribute to this accumulation (Muller and Connor, 1991, 1992; Tsunokawa and Ross, 1997; Beier and Barish, 2000). However, our results suggest that the stimulation of R-type Ca^{2+} currents also might contribute to the muscarinic-mediated intracellular Ca^{2+} accumulations.

All three T-type VSCC subunits (α1G, α1H, and α1I) are expressed in hippocampal pyramidal neurons (Taylor et al., 1999). However, their modulation and functional impact on hippocampal pyramidal neurons are not yet known. Compared with HVA channels, T-type VSCCs are more metabolically stable and are less likely to be modulated (Huang et al., 2005). Muscarinic activation has been reported to increase, decrease, or not affect the T-type Ca^{2+} currents, depending on the cell type and experimental conditions (Yunker, 2003). In the present study T-type Ca^{2+} currents in hippocampal pyramidal neurons were not affected by muscarinic stimulation.

Muscarinic stimulation is known to suppress K⁺ channels, such as M channels (Brown and Adams, 1980; Halliwell and Adams, 1982), thereby enhancing neuronal excitability. However, in the present study the enhancement of R-type Ca^{2+} currents is not attributable to K⁺ channel depression. High concentrations of potassium channel blockers were applied both intracellularly and extracellularly (see Materials and Methods), and most K⁺ currents were observed to be blocked. When the HVA Ca^{2+} currents were blocked first with Cd^{2+} (1 mM), muscarinic activation did not affect the residue K⁺ currents (data not shown). In addition, the repetitive R-type Ca^{2+} spike firing we observed in carbachol was not elicited with K⁺ channel inhibitors. Furthermore, T-type Ca^{2+} currents were found to be unaltered by carbachol application. Under normal physiological conditions it is likely that the suppression of K⁺ channels and enhancement of R-type VSCCs both contribute to the muscarinic stimulation of neuronal excitability.

A concern for this type of study is the possibility of poor voltage control during voltage-clamp experiments. To achieve well clamped currents, we applied high concentrations of K⁺ channel blockers both intracellularly and extracellularly (Colino and Halliwell, 1993), and we used younger animals (13–16 d) so that the calcium currents would be smaller and the dendritic processes less extensive. Also, large electrode tips were used (3–5 MΩ), and only cells with access resistances <20 MΩ were included in the study. The voltage clamp appeared to be sufficient in most experiments because there was a graded turn-on of the currents, and channel activation properties of the currents were similar to those observed in acutely isolated neurons (Foehring et al., 2000; Sochivko et al., 2003).

R-type spikes and theta oscillations
Muscarinic receptor stimulation in the hippocampus in vitro generates robust oscillations, which share a common frequency with theta rhythm observed in vivo. The activation of oscillatory intrinsic conductances contributes importantly to theta current generation (Buzsaki, 2002). Voltage-dependent oscillations have been described in the somata (Leung and Yim, 1991) and dendrites of pyramidal neurons (Kamondi et al., 1998). During theta induction the somatic membrane hyperpolarizes, whereas den-
drites depolarize. When the dendritic depolarization is sufficiently strong, the resonant property of the membrane leads to a HYA Ca\(^{2+}\) spike-dependent self-sustained oscillation in the theta frequency range (Kamondi et al., 1998). Nickel ions selectively abolish dendritic calcium spikes via blocking R- and/or T-type VSCCs (Gillies et al., 2002; Isomura et al., 2002). Here we have demonstrated that carbachol-enhanced, Ni\(^{2+}\)-sensitive R-type Ca\(^{2+}\) spikes can fire repetitively at theta frequencies. Given that T-type VSCCs are not affected by carbachol, our results suggest that R-type VSCCs contribute to the amplification of the theta oscillations.

Based on pharmacological sensitivity, two types of theta could be distinguished: atropine-sensitive and atropine-resistant (Kramis et al., 1975; Buzsaki, 2002). Our results show that Ni\(^{2+}\) blocked carbachol-induced spontaneous theta burst oscillations. This agrees with a previous study demonstrating that Ni\(^{2+}\) (100 \(\mu\)M) blocked mGluR-activated but atropine-resistant theta oscillations (Gillies et al., 2002). In this previous study Ni\(^{2+}\)-dependent spikes were generated in the distal dendrites at theta frequencies. We found that the mGluR agonist trans-ACPD (trans-1-amino cyclopentane-1,3-di carboxylic acid; 50 \(\mu\)M) and DHPG (dihyd rophenyl glycol; 50 \(\mu\)M) resulted in enhanced R-type Ca\(^{2+}\) spiking similar to that observed with carbachol (data not shown) (Kuzmiski and MacVicar, 2003). These results suggested that a similar contribution of R-type VSCCs to both atropine-sensitive and atropine-resistant forms of theta might exist.

In the hippocampus Ca\(^{2+}\) spikes are generated during highly synchronous excitatory input that is associated with behavior \textit{in vivo}. The ability of carbachol-enhanced R-type Ca\(^{2+}\) spikes to resonate at theta frequencies suggests that it is an intrinsic property of the neuronal membrane that plays a key role in the generation of neuronal network oscillations. The resonance property of R-type VSCCs also may be important for synaptic plasticity induced by theta burst stimulation (Ito et al., 1995), the enhanced induction of theta during cholinergic stimulation (Huerta and Lisman, 1993), and the formation of accurate spatial memory (Kubota et al., 2001).

References

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