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Muscarinic Induction of Hippocampal Gamma Oscillations Requires Coupling of the M1 Receptor to Two Mixed Cation Currents

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Summary

Oscillatory network activity at γ frequencies is assumed to be of major importance in cortical information processing. Whereas the synaptic mechanisms of γ oscillations have been studied in detail, the ionic currents involved at the cellular level remain to be elucidated. Here we show that in vitro γ oscillations induced by muscarine require activation of M1 receptors on hippocampal CA3 pyramidal neurons and are absent in M1 receptor-deficient mice. M1 receptor activation depolarizes pyramidal neurons by increasing the mixed Na^+/K^+ current I_h and the Ca^{2+}-dependent nonspecific cation current I_{cat}, but not by modulation of I_M. Our data provide important insight into the molecular basis of γ oscillations by unequivocally establishing a novel role for muscarinic modulation of I_h and I_{cat} in rhythmic network activity.

Introduction

Both γ oscillations (20–80 Hz) in transient assemblies of neurons as well as muscarinic cholinergic neurotransmission play an important role in hippocampal information processing (Morris et al., 1982; Gray et al., 1989; Steriade et al., 1991; Hasselmo et al., 1992; Bliss and Collingridge, 1993; Singer, 1993; Bragin et al., 1995). Blockade of muscarinic receptors or lesions of the cholinergic septohippocampal pathway produces memory and attentional deficits (Bartus et al., 1982). In addition, central cholinergic receptor dysfunction has been suggested to be involved in memory loss and other cognitive deficits in schizophrenia as well as Alzheimer's and Parkinson's diseases (Coyle et al., 1983; Griffiths et al., 1994; Haroutunian et al., 1994). Intriguingly, evidence from magnetoencephalograms suggests that neurodegenerative diseases like Alzheimer's, in which memory formation is impaired, are associated with the disruption of γ oscillations (Ribary et al., 1991). Furthermore, γ oscillations and their synchronization are prominent during states of focused attention or when subjects are engaged in visual discrimination tasks, which require feature binding mechanisms and involve short-term memory (Tallon-Baudry et al., 1997).

In the in vitro hippocampus, persistent γ oscillations are reliably induced by muscarinic receptor activation (Fisahn et al., 1998). Recent pharmacological studies have suggested that it is the activation of the M1 muscarinic receptor that is important for the induction of γ oscillations (Fisahn et al., 1998). However, the receptor subtype selectivity of the available muscarinic ligands is limited (Caulfield, 1993). Muscarinic-cholinergic agonists typically have several excitatory actions on hippocampal neurons. These include suppression of three separate K^+ conductances (the voltage- and time-dependent potassium current I_M, the slow Ca^{2+}-activated K^+ current I_{AHP}, and the time- and voltage-independent leakage K^+ current) (Halliwell and Adams, 1982; Lancaster and Adams, 1986; Madison et al., 1987) and potentiation of two mixed cation currents (the hyperpolarization-activated current I_h and a Ca^{2+}-dependent, nonspecific cation conductance I_{cat}) (Brown and Adams, 1980; Halliwell and Adams, 1982; Colino and Halliwell, 1993). However, recent evidence has suggested that muscarinic suppression of hippocampal potassium conductances is unchanged in mice lacking M1 receptors (Rouse et al., 2000; but see Hamilton et al., 1997). Whether M1 receptors selectively modulate either of the two mixed cation conductances remains untested.

To conclusively answer the question, which of the five muscarinic receptors (M1–M5) expressed in hippocampus (Levey et al., 1995) is involved in the generation of this rhythmic network activity, we generated a series of muscarinic receptor-deficient mice. Using these mice, we analyzed the hitherto completely unexplored link between M1 muscarinic receptors and intrinsic conductances expressed in single hippocampal CA3 pyramidal neurons and their contribution to the induction of γ oscillations. Our data demonstrate that selective activation of M1 muscarinic receptors modulates both the mixed Na^+/K^+ current I_h and the Ca^{2+}-dependent nonspecific cation current I_{cat} and is required for induction of hippocampal γ oscillations. Conversely, the absence of M2–M5 muscarinic receptors leaves γ oscillations unaffected.

Results

Generation of M1 Muscarinic Receptor-Deficient Mice

Homozygous M1 receptor mutant mice ($M1^{-/-}$ mice; Figures 1A and 1B) were obtained with the expected Mendelian frequency (see Experimental Procedure) and did not differ from their wild-type (wt) littermates in overall health, fertility, or longevity. Immunoprecipitation studies using membranes prepared from mouse hippocampus ($n = 4$; Figure 1C) and several other brain regions ($n = 4$; data not shown) confirmed that $M1^{-/-}$

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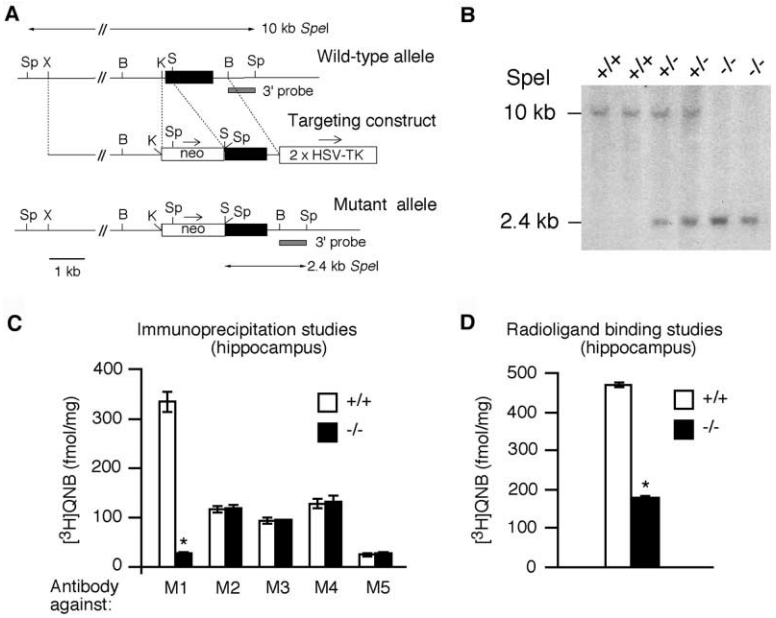

Figure 1. Targeted Disruption of the Mouse M1 Muscarinic Receptor Gene

(A) Structure of the wild-type allele, targeting vector, and mutated allele. The M1 receptor coding region is represented by a closed box. The 3′ probe that was used for Southern analysis and the sizes of the restriction fragments detected by this probe are indicated. B, *Bam*HI; K, *Kpn*I; S, *Sac*I; Sp, *Spe*I; and X, *Xba*I.

(B) Southern blot analysis of *Spe*I-digested genomic DNA from mouse tail DNA prepared from F2 pups generated by intermating of F1 heterozygotes. The 10 kb and 2.4 kb bands represent the wt and mutant M1 receptor alleles, respectively.

(C) Membranes prepared from mouse hippocampus were incubated with 2 nM of [^{3}H]QNB. [^{3}H]QNB-labeled muscarinic receptors were solubilized and immunoprecipitated with M1–M5 receptor-specific antisera (Gomeza et al., 1999a, 1999b).

(D) Membranes prepared from mouse hippocampus were incubated with a saturating concentration (2 nM) of the nonselective muscarinic antagonist, [^{3}H]QNB ($n = 4$; *, $p < 0.05$).

mice lacked functional M1 receptor protein (only a weak background signal was observed with tissues from M1$^{-/-}$ mice; Figure 1C). These data also demonstrate that the absence of M1 receptors did not lead to compensatory changes in the expression levels of the remaining four muscarinic receptor subtypes M2–M5 ($n = 4$; Figure 1C). Radioligand binding studies using the nonselective muscarinic antagonist [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) revealed a drastic reduction (by ~50%–60%) in the total number of muscarinic binding sites in the hippocampus of M1$^{-/-}$ mice ($n = 4$; Figure 1D). Similar results were obtained with several other mouse forebrain tissues, including cerebral cortex and striatum ($n = 4$; data not shown). These findings are consistent with previous studies indicating that M1 receptors are abundantly expressed in higher brain regions (Caulfield, 1993; Levey et al., 1995).

Muscarine-Induced γ Oscillations Are Absent in M1$^{-/-}$ Hippocampus

Initially, we employed extracellular field recordings to test for differential responses in in vitro hippocampi of wt and M1$^{-/-}$ mice to agents that induce γ oscillations. Bath application of the nonselective muscarinic receptor agonist muscarine (10–50 μM; $n = 5$; Figures 2A and 2B) or the kainate receptor agonist kainate (100 nM; $n = 5$; Figure 2B) induced γ oscillations in area CA3 of wt mouse hippocampal slices (Buhl et al., 1998; Fisahn et al., 1998). Strikingly, muscarine-induced activity was totally abolished in preparations from M1$^{-/-}$ mice ($n = 7$; Figures 2C and 2D). However, subsequent application of kainate (100 nM) to hippocampal slices of M1$^{-/-}$ mice confirmed the ability of the neuronal network to produce γ oscillations ($n = 3$; Figures 2C and 2D). In contrast, muscarine (20 μM) was able to induce wt-like hippocampal γ oscillations in mutant mice lacking M2 (Gomeza et al., 1999a), M3 (Yamada et al., 2001b), M4 (Gomeza et al., 1999b), or M5 (Yamada et al., 2001a) muscarinic receptors ($n = 3$ each; Figure 3). Therefore, activation of the M1 muscarinic receptor, but not M2–M5, is necessary for the induction of muscarinic-cholinergic γ oscillations in the hippocampal slice.

To elucidate the effects of muscarine on single pyramidal neurons, we next carried out whole-cell, patch-clamp experiments in the current-clamp configuration ($V_m = V_{rmp} = -62.0 \pm 1.3$ mV). In wt pyramidal neurons, 20 μM muscarine, a concentration that robustly induced extracellular γ oscillations, caused a membrane potential depolarization of 15.7 ± 1.4 mV ($n = 9$; Figure 4A). This was accompanied by a significant increase in action potential firing frequency from 0.6 ± 0.3 to 6.1 ± 0.8 Hz ($n = 9$; $p < 0.0001$). In contrast, M1$^{-/-}$ pyramidal neurons showed only a modest muscarine-induced depolarization (7.5 ± 1.2 mV; $n = 6$), which was delayed in onset compared to wt. Similar to wild-type, action potential firing frequency increased 10-fold from 0.2 ± 0.2 to 2.4 ± 0.7 Hz ($p = 0.0021$; Figure 4A).

As stated earlier, muscarinic-cholinergic agonists have several excitatory actions on hippocampal neurons. These include suppression of three separate K^+ conductances (the voltage- and time-dependent potassium current I_M, the slow Ca^{2+}-activated K^+ current I_{AHP}, and the time- and voltage-independent leakage K^+ current) (Halliwell and Adams, 1982; Lancaster and Adams, 1986; Madison et al., 1987) and potentiation of two mixed cation currents (the hyperpolarization-activated current I_h and a Ca^{2+}-dependent nonspecific cation conductance I_{cat}) (Brown and Adams, 1980; Halliwell and Adams, 1982; Colino and Halliwell, 1993). Recent evidence has shown, however, that muscarinic suppression of hippocampal potassium conductances is unchanged in mice lacking M1 receptors (Rouse et al., 2000; but see Hamilton et al., 1997). That muscarinic modulation of hippocampal potassium conductances persists in M1$^{-/-}$ animals, presumably rules out an essential role for these conductances in γ oscillation induction described here.

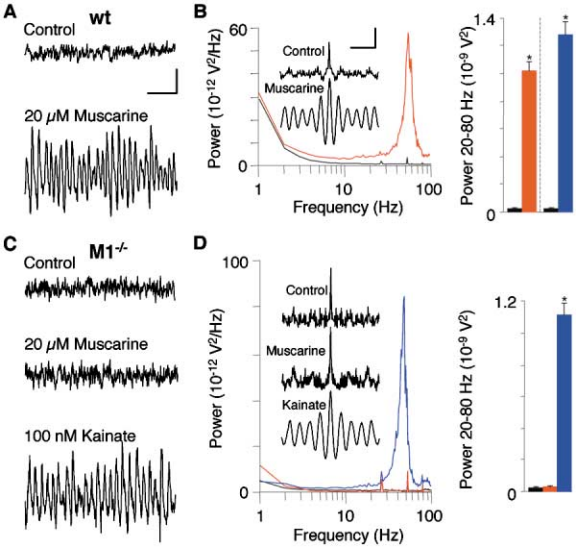

Figure 2. Disruption of Muscarine-Induced γ Oscillations in M1$^{-/-}$ Hippocampal Slices

(A) Example traces of extracellular field recordings from stratum pyramidale of the CA3 area. In wt slices, no rhythmic network activity is seen in control conditions (no drug). Bath application of muscarine induces γ oscillations. Scale bars, 50 μV and 100 ms.

(B) Power spectra of control (black) and muscarine-induced oscillations (red). The inset shows autocorrelograms of the traces in (A). Scale bars, 0.5 and 50 ms. The histogram shows the integrated power from 20 to 80 Hz for control conditions (black) and after application of either muscarine (red; n = 6) or kainate (blue; n = 3).

(C) In M1$^{-/-}$ slices, muscarine fails to induce γ oscillations, whereas they can still be induced by kainate.

(D) Power spectra (control, black; muscarine, red; and kainite, blue) and autocorrelograms (inset) of the traces shown in (C). The histogram shows the integrated power from 20 to 80 Hz for control conditions (black) and after application of muscarine (red) and kainate (blue; n = 6; *, $p < 0.05$).

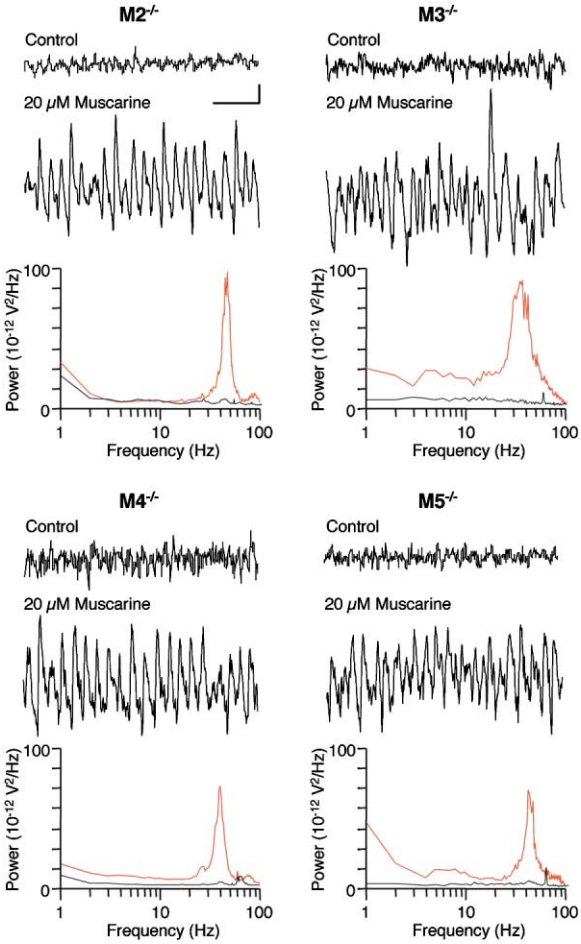

Figure 3. Wild-Type-like γ Oscillations in M2$^{-/-}$, M3$^{-/-}$, M4$^{-/-}$, and M5$^{-/-}$ Hippocampal Slices

Example traces of extracellular field recordings from stratum pyramidale of the CA3 area. No rhythmic network activity is seen in control conditions (no drug). Bath application of muscarine induces γ oscillations. Scale bars, 50 μV and 100 ms. Power spectra of control (black) and muscarine-induced oscillations (red) are shown below the original traces.

Consequently, we next determined whether muscarinic modulation of either of the mixed cation currents was linked to M1 receptor activation-induced γ oscillations.

The M1 Receptor Modulates the Nonspecific Cation Current

Under voltage-clamp configuration at potentials close to the resting membrane potential ($V_h = -60$ mV), muscarine (20 μM) caused a marked inward current of 52.8 ± 2.3 pA in single wt pyramidal neurons (n = 10). In contrast, only a small residual inward current (5.7 ± 0.7 pA) was observed in their M1$^{-/-}$ counterparts (n = 13; Figure 4B). The same experiment was repeated in anatomically identified stratum radiatum interneurons in area CA3 of wt (n = 8) and M1$^{-/-}$ preparations (n = 10). In contrast to pyramidal neurons, no significant difference could be observed between muscarine-induced inward currents in wt (13.5 ± 13.5 pA) and M1$^{-/-}$ interneurons (18.4 ± 17.5 pA; data not shown). This coroborates anatomical data by Levey et al. (1995) that demonstrated expression of the M1 musacrinic receptor in pyramidal neurons, but not inhibitory interneurons.

Previous studies have shown that a significant portion of the muscarinic receptor-induced inward current in CA1 pyramidal neurons results from activation of a Ca^{2+}-dependent nonspecific cation current (Colino and Halliwell, 1993) and can be blocked by agents that prevent Ca^{2+} influx or by replacement of extracellular Na^+ ions (Colino and Halliwell, 1993). In wt neurons, blockade of Ca^{2+} influx by Cd^{2+} (500 μM) decreased the inward current by 59.5% ± 12.3% (n = 3). Moreover, lowering the extracellular Na^+ concentration to 25 mM by substitution of NaCl with N-methyl-D-glucamine (Colino and Halliwell, 1993) eliminated the muscarine-induced inward current. Instead, a prominent outward current was observed in wt pyramidal neurons (19.2 ± 4.7 pA, n = 3; Figure 4C). This outward current likely resulted from the shift in reversal potential for I_{cat} (as well as the hyperpolarization-activated current I_h) under conditions of reduced extracellular Na^+ ions. These observations are consistent with muscarinic activation of a Ca^{2+}-dependent nonspecific cation current (Colino and Halliwell, 1993). In M1$^{-/-}$ pyramidal neurons, despite a near 10-fold reduction in the inward current amplitude induced

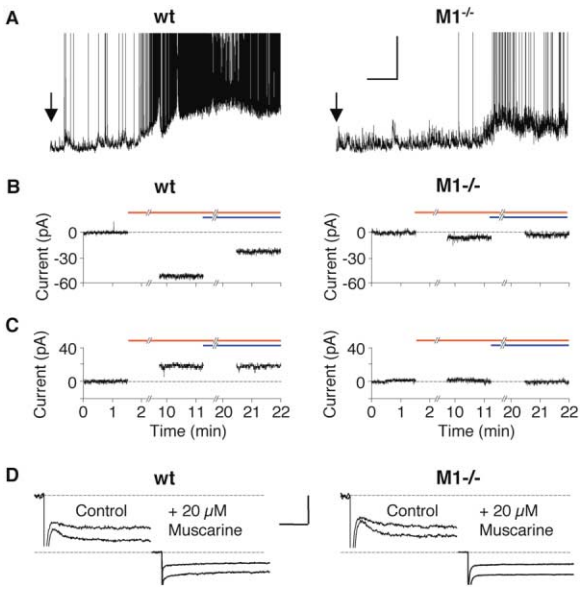

Figure 4. Muscarinic Modulation of I_{cat} but Not I_M Is Lost in $M1^{-/-}$ Pyramidal Neurons

(A) In current clamp ($V_m = -62.0 \pm 1.3$ mV), bath application of 20 μM muscarine (arrow) leads to a differential depolarization and increase in action potential firing frequency of wt and $M1^{-/-}$ CA3 pyramidal neurons. Action potentials are truncated for figure clarity. Scale bars, 20 mV and 20 s.

(B) In voltage clamp ($V_h = -60$ mV), 20 μM muscarine (indicated by red bar) leads to a differential increase in inward current in wt and $M1^{-/-}$ pyramidal neurons. The inward current is reduced by subsequent application of 500 μM Cd^{2+} (blue bar). Dotted lines show the predrug holding current.

(C) As in (B) but with $[Na^+]_o$ lowered to 25 mM. Under these conditions, muscarine causes an outward current in wt pyramidal neurons that is unaffected by application of Cd^{2+}. In $M1^{-/-}$ pyramidal neurons, no significant change in current is observed.

(D) From a holding potential of −40 mV, a 1 s test pulse to −60 or −80 mV in control conditions results in a relaxation current typical for I_M deactivation in both wt and $M1^{-/-}$ pyramidal neurons. Application of 20 μM muscarine leads to an increase in holding current and a block of the time-dependent current I_M in both wt and $M1^{-/-}$ pyramidal neurons (averages of 5 pulses at 0.1 Hz are shown; 2 mM Cs^+ present throughout to block I_h). Scale bars, 20 pA and 100 ms.

by muscarine (~50 pA in wt versus ~6 pA in $M1^{-/-}$), Cd^{2+} blocked the inward current to a similar degree as in wt (56.1% ± 36.8%; n = 3; Figure 4B). In addition, in the presence of lowered $[Na^+]_o$, no significant change in current was observed upon application of muscarine (n = 3; Figure 4C). These data indicate that while muscarinic activation of I_{cat} occurs mainly via M1 receptors, other muscarine receptors may also activate a similar conductance albeit to a lesser degree in $M1^{-/-}$. These data demonstrate that a muscarine-induced augmentation of I_{cat} contributes a significant portion (but not all) of the tonic depolarization of wt pyramidal neurons observed in current-clamp experiments. In contrast, the depolarizing drive resulting from muscarine receptor activation in $M1^{-/-}$ pyramidal neurons is much smaller.

The M1 Receptor Does Not Modulate the M Current

Additional excitatory drive to pyramidal neurons may arise from muscarinic suppression of the time- and voltage-dependent potassium current I_M. Although a previous study has shown that the M1 receptors do not increase CA1 pyramidal neuron excitability via suppression of I_M (Rouse et al., 2000), recordings from sympathetic ganglion neurons have shown a link between M1 receptors and I_M (Hamilton et al., 1997). To directly rule out a role for I_M modulation, CA3 pyramidal neurons were next voltage clamped at −40 mV and hyperpolarizing test pulses to −60 and −80 mV were applied (1 s duration, 5 times at 0.1 Hz; 2 mM Cs^+ present throughout to block I_h). This protocol resulted in voltage- and time-dependent relaxation currents typical for I_M deactivation that were apparent in control traces of both wt (n = 6) and $M1^{-/-}$ pyramidal neurons (n = 6; Figure 4D). At a holding potential of −40 mV, application of muscarine (20 μM) caused both an increase in holding current (wt, 38.1 ± 3.3 pA; $M1^{-/-}$, 42.3 ± 4.8 pA) and suppression of the time-dependent relaxation, consistent with a block of I_M (Figure 4D). Digital subtraction of the current resulting from the −60 mV test pulse in the presence of muscarine from the current activated under control conditions allowed inspection of the "pure" I_M component suppressed by muscarine (data not shown). The charge transfer associated with this current component was 22.0 ± 2.7 pC in wt (n = 6) and 61.0 ± 4.5 pC in $M1^{-/-}$ pyramidal neurons (n = 6). It is interesting to note that although muscarine suppressed I_M in $M1^{-/-}$ pyramidal neurons, the charge transfer associated with I_M in the knockout was significantly greater than that observed in experiments from wt. The reasons for this are unclear, but we would speculate that the differing amounts of I_M in wt and $M1^{-/-}$ pyramidal neurons may result from a different composition or reorganization of the M channels in the $M1^{-/-}$ mouse. Although no compensatory increases in expression of the other muscarinic receptors were observed in the $M1^{-/-}$ animals (Figure 1C), perhaps the transduction mechanisms of the remaining channels are different. However, despite the larger M current in $M1^{-/-}$, suppression of this conductance is unable to significantly depolarize CA3 pyramidal neurons at their resting membrane potential (Figure 4A) and, more importantly, does not result in γ oscillatory activity (Figure 2).

Subsequent application of the selective I_M antagonist linopirdine (10 μM) after muscarine (n = 6) further increased the amount of I_M charge transfer removed (wt, 29.2 ± 3.9 pC; $M1^{-/-}$, 78.1 ± 5.7 pC), indicating that at a concentration of 20 μM, muscarine's suppression of I_M was submaximal in both wt and $M1^{-/-}$ (data not shown). In addition, prior incubation of slices in linopirdine (10 μM) prevented modulation of I_M by muscarine (no measurable removal of charge transfer) in both wt and $M1^{-/-}$ pyramidal neurons (n = 4 each; data not shown). These data demonstrate that, similar to CA1 pyramidal neurons, muscarinic modulation of I_M in CA3 pyramidal neurons does not require activation of M1 receptors (Rouse et al., 2000).

The M1 Receptor Modulates the Hyperpolarization-Activated Cation Current

The hyperpolarization-activated mixed cation current I_h is present in both cardiac and neuronal tissues and plays important functional roles, including the generation of

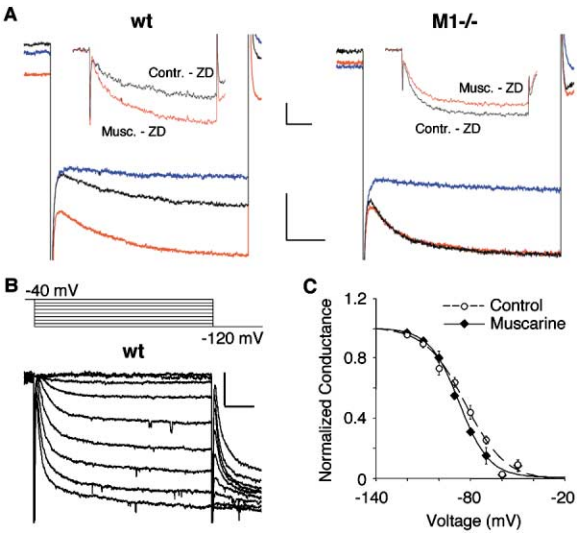

Figure 5. Muscarine Modulates I_h in Wild-Type but Not $M1^{-/-}$ Pyramidal Neurons

(A) Hyperpolarizing test pulses from a holding potential of −70 to −100 mV (1 s duration) under control conditions (black) activate I_h in both wt and $M1^{-/-}$ pyramidal neurons. Application of 20 μM muscarine (red) leads to an increase in both the holding current and the time-dependent current I_h in wt pyramidal neurons. The selective I_h antagonist ZD7288 (100 μM) blocks the time-dependent current and partially restores the holding current (blue; averages of 10 pulses at 0.1 Hz are shown; 1 mM Ba^{2+} present throughout to block I_M, I_{leak}, and the inwardly rectifying K^+ channel K_{IR}). Scale bars (lower), 40 pA and 200 ms. Digitally subtracted traces (insets, baselined) show the net h current activated by the hyperpolarizing test pulse before and after the application of muscarine. Scale bars (upper), 20 pA and 200 ms.

(B) Leak-corrected current family recorded in control conditions in wt pyramidal neurons showing relaxation responses typical for I_h (1 mM Ba^{2+} present throughout to block I_M, I_{leak}, and the inwardly rectifying K^+ channel K_{IR}). Scale bars, 20 pA and 500 ms.

(C) Activation curves were constructed from current families in control conditions (B) and after the application of 20 μM muscarine and fitted using the Boltzman function (Maccaferri and McBain, 1996).

pacemaker current in the heart (Brown et al., 1979), repetitive firing in hippocampal neurons (Maccaferri et al., 1993; Maccaferri and McBain, 1996; Santoro et al., 2000), and oscillatory rhythms in thalamo-cortical neuronal circuits (Pape and McCormick, 1989; Soltesz et al., 1991; Pape, 1996). To investigate a possible link between M1 receptor activation and modulation of I_h, pyramidal neurons were held at −70 mV and test pulses to −100 mV (1 s duration) were delivered (5 times at 0.1 Hz; 1 mM Ba^{2+} present throughout to block I_M, I_{leak}, and the inwardly rectifying K^+ channel K_{IR}) (Maccaferri et al., 1993). I_h was observed in control traces of both wt ($n = 6$) and $M1^{-/-}$ pyramidal neurons ($n = 5$) as a slowly activating inward current (Maccaferri and McBain, 1996; Ludwig et al., 1998; Santoro et al., 2000) (Figure 5A). At this test pulse, the kinetics of I_h activation were best fit by the sum of two exponentials (mean $\tau_{fast} = 91 \pm 18$ ms, 81% of the total current; mean $\tau_{slow} = 433 \pm 127$ ms, 19% of the total current; $n = 6$), consistent with previous reports (Santoro et al., 2000).

Application of muscarine (20 μM) caused an increase in magnitude of I_h activated in wt but not $M1^{-/-}$ pyramidal neurons and differentially increased the holding current of wt (32.9 ± 2.2 pA) and $M1^{-/-}$ pyramidal neurons (17.3 ± 1.6 pA; Figure 5A). External application of the selective I_h antagonist ZD7288 (100 μM) (BoSmith et al., 1993; Maccaferri and McBain, 1996) markedly reduced the amplitude and time dependence of the inward current activated by a test pulse to −100 mV (Figure 5A). In addition, a decrease in the holding current was observed in wt pyramidal neurons (12.5 ± 3.2 pA) but not in their $M1^{-/-}$ counterparts (−0.8 ± 4.3 pA). Digital subtraction of the records obtained in the presence of ZD7288 from the traces obtained in control conditions and in the presence of muscarine allowed inspection of the "pure" I_h component modulated by muscarinic receptor activation (Figure 5A). Muscarine significantly increased the charge transfer associated with I_h in wt pyramidal neurons ($n = 6$) from 28.4 ± 3.1 pC under control conditions to 40.3 ± 3.7 pC ($p = 0.049$; Figure 5A). No such effect was observed in $M1^{-/-}$ pyramidal neurons ($n = 5$); in contrast, muscarine significantly *decreased* the I_h charge transfer from 33.0 ± 2.9 pC under control conditions to 27.8 ± 2.6 pC ($p = 0.032$; Figure 5A). Prior application of ZD7288 prevented detection of any time-dependent current component, and under these conditions, muscarine failed to modulate currents activated by hyperpolarizing test potentials ($n = 3$; data not shown). These results demonstrate that the muscarinic activation of I_h is linked to the M1 receptor.

Mechanism of I_h Modulation by Muscarinic Activation of M1 Receptors

A number of neuromodulators increase I_h by shifting its voltage dependence of activation toward more depolarized potentials (McCormick and Pape, 1990) and/or by increasing its maximal conductance (Gasparini and DiFrancesco, 1999). To test if muscarinic activation of M1 receptors affects the I_h activation curve, a two-step voltage protocol was employed (Maccaferri and McBain, 1996) (Figure 5B). The first step elicited I_h in the range −40 to −120 mV (3 s duration) from a V_h of −40 mV. From each initial test potential, I_h was subsequently fully activated with a second step to −120 mV (3 s duration; 1 mM Ba^{2+} present throughout to block I_M, I_{leak}, and the inwardly rectifying K^+ channel K_{IR}; Figure 5B). The differences between the resulting relaxation currents and the maximal current available at −120 mV after normalization is directly related to the degree of I_h activation in the first test step. These values were used to construct I_h activation curves before and after the application of 20 μM muscarine (fitted by a Boltzman function [Maccaferri and McBain, 1996]; $n = 8$ each; Figure 5C). The data clearly show that there is no significant shift of the I_h activation curve in the presence of muscarine (control, $V_{1/2} = -83.4 \pm 1.3$ mV, $k = -11.7 \pm 1.2$; muscarine, $V_{1/2} = -87.4 \pm 0.6$ mV, $k = -9.4 \pm 0.5$; $p < 0.05$; Figure 5C).

We next investigated whether muscarine receptor activation increased the maximal I_h conductance using a slow voltage ramp procedure (Gasparini and DiFrancesco, 1999) (Figure 6A). In control conditions, pyramidal neurons were held at −40 mV and then ramped (1.33 mV/s, 5 times) to −120 mV (500 μM Cd^{2+} present throughout to block I_{cat}; present throughout was 1 mM

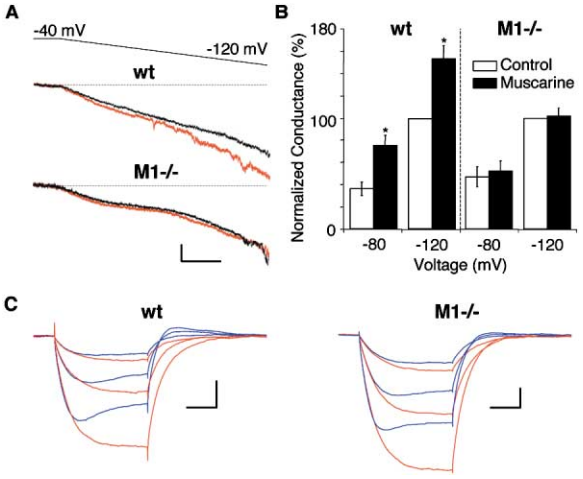

Figure 6. Muscarine Increases the I_h Conductance in Wild-Type but Not $M1^{-/-}$ Pyramidal Neurons

(A) Leak-corrected current responses to a voltage ramp protocol (1.33 mV/s) (Gasparini and DiFrancesco, 1999). The addition of muscarine increases I_h in wt but not $M1^{-/-}$ pyramidal neurons (control, black; 20 μM muscarine, red; 500 μM Cd^{2+} present throughout to block I_{cat}; 1 mM Ba^{2+} present throughout to block I_M, I_{leak}, and the inwardly rectifying K^+ channel K_{IR}). Scale bars, 50 pA and 10 s.

(B) Conductance values at voltages where I_h is partially or fully activated were calculated from the ramp experiments in (A). Data were normalized to the I_h conductance at −120 mV under control conditions ($n = 6$; *, $p < 0.05$).

(C) Current pulses (−20, −50, and −100 pA; 300 ms; 0.1 Hz; averages of 20 pulses shown) were delivered to wt and $M1^{-/-}$ pyramidal neurons ($V_m = -61.4 \pm 0.8$ mV). The resulting hyperpolarization (−5, −15, and −30 mV) revealed a voltage- and time-dependent "sag" reminiscent of I_h activation (blue; 1 μM TTX present). The addition of the I_h antagonist ZD7288 (100 μM) abolished the time-dependent "sag" (red), indicating significant I_h activation at voltage excursions close to the resting membrane potential. Scale bars, 10 mV and 200 ms.

Ba^{2+} to block I_M, I_{leak}, and the inwardly rectifying K^+ channel K_{IR}). This was repeated following addition of 20 μM muscarine and again after block of I_h by 100 μM ZD7288. To correct for leakage current, the recording in the presence of ZD7288 was subtracted from the recordings in control conditions and in the presence of muscarine (Figure 6A). By this method, muscarine clearly increased I_h across the entire voltage range in wt pyramidal neurons ($n = 5$) but not in their $M1^{-/-}$ counterparts ($n = 6$). The conductance values at the half-activation (−80 mV) and full-activation voltages of I_h (−120 mV) were calculated and normalized to the maximal conductance observed in control. In wt pyramidal neurons ($n = 5$), muscarine increased the I_h conductance at −80 mV from 37% ± 8% to 75% ± 10% ($p < 0.03$) and at −120 mV to 154% ± 14% of control ($p < 0.03$). In contrast, in $M1^{-/-}$ pyramidal neurons ($n = 6$), muscarine failed to significantly alter the I_h conductance both at −80 mV (from 47% ± 10% to 51% ± 9%; $p > 0.05$) and −120 mV (from 100% to 102% ± 7%; $p > 0.05$; Figure 6B). Therefore, muscarinic activation of the M1 receptor augments I_h by increasing its conductance.

I_h Is Active at Resting Membrane Potentials

Given that I_h is predominantly activated at more hyperpolarized membrane potentials, we next addressed the question of whether I_h was activated at potentials close to the resting membrane potential to allow it to contribute to pyramidal neuron γ oscillatory activity. Previous studies have shown that a significant amount of I_h is available at resting membrane potentials (Maccaferri et al., 1993; Maccaferri and McBain, 1996; Magee, 1998). Consistent with these previous reports, Figure 5C shows that ~10% of the I_h conductance is available at −60 mV. Under current-clamp conditions, with cells held at the resting membrane potential ($V_m = -61.4 \pm 0.8$ mV), small current pulses (−20, −50, and −100 pA; 300 ms; 0.1 Hz) were delivered to both wt and $M1^{-/-}$ pyramidal neurons to determine whether the I_h conductance was active. The resulting hyperpolarization (−5, −15, and −30 mV) revealed a voltage- and time-dependent "sag" reminiscent of I_h activation (1 μM TTX present). The addition of the I_h antagonist ZD7288 (100 μM) abolished the time-dependent "sag" ($n = 4$ each; Figure 6C) observed in response to all current pulse amplitudes. These data confirm that significant I_h is activated by small voltage excursions around the resting membrane potential of CA3 pyramidal neurons. Finally, in Figure 4B we demonstrated that ~60% of the inward current activated at resting membrane potentials by muscarine was carried by I_{cat}. We repeated this experiment to determine what fraction of this current was carried by I_h. Following block of the I_{cat} by Cd^{2+}, addition of ZD7288 (100 μM) blocked ~10% of the total current observed in control (mean current block 9.2% ± 4.7%; $n = 6$; data not shown). These data demonstrate that both I_h *and* I_{cat} contribute to the inward current generated by muscarine application at resting membrane potentials.

Effect of I_h and I_M Antagonists on γ Oscillations

In the above experiments, we have shown that M1 receptor activation by musacrine modulates I_{cat} and I_h, but not I_M. If the modulation of I_{cat} and I_h is of importance for extracellular γ oscillations, then blocking these conductances should lead to an alteration of the extracellular activity. Likewise, γ oscillations should remain unaffected by a block of I_M.

Indeed, we could confirm a role for I_h, and rule out a role for I_M, in γ oscillations employing extracellular field recordings in wt mice. Superfusion of naïve slices with a maximal concentration of the I_M antagonist linopirdine (10 μM) failed to induce rhythmic network activity, whereas subsequent addition of 20 μM muscarine, in the continued presence of linopirdine, induced γ oscillations ($n = 3$; Figures 7A and 7B). Similarly, application of linopirdine (10 μM) following the induction of γ oscillations by muscarine ($n = 3$; data not shown) failed to alter rhythmic activity in the γ frequency band. These data demonstrate that modulation of I_M is neither necessary nor sufficient for muscarinic induction of γ oscillations. In contrast, blockade of I_h by ZD7288 (100 μM) eliminated established muscarine-induced γ oscillations ($n = 3$; Figures 7C and 7D). Similarly, preincubating hippocampal slices in ZD7288 (100 μM) prevented subsequent induction of extracellular γ oscillations ($n = 3$; data not shown) by muscarine (20 μM). These data clearly indicate that muscarinic receptor-mediated augmentation of I_h is essential for the induction and maintenance of hippocampal γ oscillations. Similar field experi-

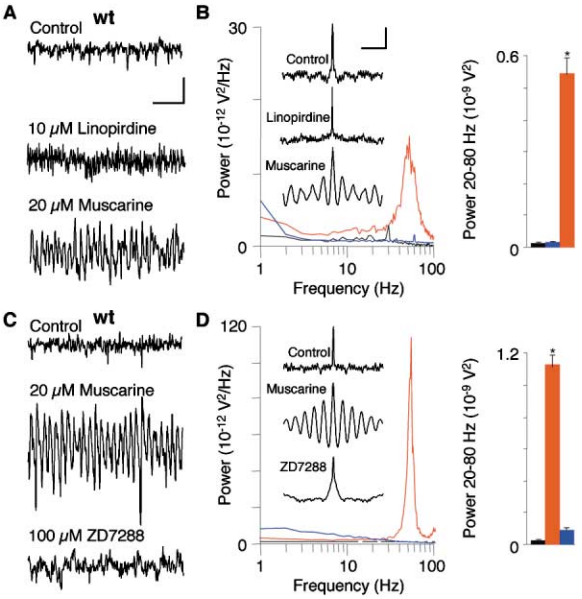

Figure 7. An I_h but Not an I_M Antagonist Disrupts Muscarine-Induced Hippocampal γ Oscillations

(A) Example traces of extracellular field recordings from stratum pyramidale of the CA3 area. Prior exposure of slices to the I_M antagonist linopirdine neither induced oscillations nor prevented muscarinic induction of γ oscillations. Scale bars, 50 μV and 100 ms.

(B) Power spectra (control, black; linopirdine, blue; and muscarine, red). The inset shows autocorrelograms of the traces in (A). Scale bars, 0.5 and 50 ms. The histogram shows the integrated power from 20 to 80 Hz for control conditions (black) and after application of linopirdine (blue) and muscarine (red; n = 6).

(C) In contrast, the I_h antagonist ZD7288 abolishes muscarine-induced γ oscillations.

(D) Power spectra (control, black; muscarine, red; and ZD7288, blue) and autocorrelograms (inset) of the traces shown in (C). The histogram shows the integrated power from 20 to 80 Hz for control conditions (black) and after application of muscarine (red) and ZD7288 (blue; n = 10; *, $p < 0.05$).

ment confirmation of a role for I_{cat} was not possible since blockade of I_{cat} by Cd^{2+}, or alteration of $[Na^+]_o$, would also impact global synaptic transmission and therefore would invariably lead to a breakdown of γ oscillations in the neuronal network.

Finally, many agents that modulate the I_h conductance do so by a PKA-dependent mechanism. To determine whether cAMP-dependent modulation of I_h was involved in muscarine-induced γ oscillations, slices from wt mice were preincubated in the PKA antagonist Rp-cAMPS (0.1 mM for 2 hr; n = 4), which inhibits cAMP production. In all experiments, this treatment failed to inhibit muscarine-induced γ oscillations in the extracellular field recording (data not shown), suggesting that M1 receptor modulation of the I_h conductance may occur via a cAMP-independent mechanism (see also Colino and Halliwell, 1993).

Discussion

Our data demonstrate that muscarinic receptor-mediated augmentation of I_h and I_{cat} is linked to the M1 receptor and is essential for the muscarinic induction of hippocampal γ oscillations. We further show that similar to CA1 pyramidal neurons (Rouse et al., 2000), I_M is not modulated by M1 muscarinic receptor activation in CA3 pyramidal neurons and neither is it essential for the mucarinic induction of γ oscillations.

Previous work in the in vitro hippocampus and neocortex uncovered the chemical synaptic mechanisms that underlie rhythmic network oscillations in the γ frequency band (Buhl et al., 1998; Fisahn et al., 1998; Fisahn, 1999). Those studies determined that in cholinergically and glutamatergically induced persistent γ oscillations, rhythmic IPSPs generated by networks of interneurons pace the activity of pyramidal neurons and curtail their EPSPs. This shunting of EPSPs prevents pyramidal neurons from reaching firing threshold and only when the IPSP has decayed sufficiently, can a pyramidal neuron fire an action potential. This mechanism enables interneurons to govern the frequency of the rhythmic network activity. The curtailing of EPSPs by IPSPs sharpens the window in which pyramidal neurons can fire action potentials and may lead to a tighter synchrony among pyramidal neurons. In addition, recent studies also emphasized the importance of electrical transmission via gap junctions in γ oscillations (Fisahn, 1999; Traub et al., 2000; Hormuzdi et al., 2001).

Traub et al. (2000) have provided a detailed computer model of carbachol-induced γ oscillations that rigorously examined conditions under which network oscillations might occur within the framework of experimentally determined properties. In this model, pyramidal neuron somatic firing was not essential for the generation of γ oscillations but instead relied on a sufficiently high rate of spontaneous action potentials generated in pyramidal cell *axons*, interconnected via gap junctions located close to the axon initial segment. Such connectivity provided a plexus of pyramidal cell axons that generated spontaneous ectopic action potentials, which then percolated throughout the axonal network. This spiking activity provided an AMPA-receptor mediated synaptic input to both principal neurons and inhibitory interneurons. In the model at least, it was the synaptic excitation of interneurons and not of pyramidal cells that was a critical component of oscillatory activity. To date, the model of Traub et al. (2000) is the only one to sufficiently combine all of the known experimental observations of carbachol-induced γ into a comprehensive picture of how the CA3 network might generate γ oscillatory activity.

How then does the present data map onto the predictions of the model provided by Traub et al. (2000)? Close inspection of the model reveals that several parameters are required for carbachol-induced γ. In the model, metabotropic cholinergic effects are modeled by (i) a tonic depolarizating conductance on pyramidal cells and inclusion of a persistent noninactivating Na^+-conductance applied selectively to the dendritic compartment; (ii) a reduction in the strength of unitary pyramid-pyramid EPSPs; and (iii) suppression of g_{Ca}, the slow Ca^{2+}-mediated afterhyperpolarization, and the leakage conductance. Finally, high frequency, ectopic axonal action potentials were generated by brief current pulses to distal axonal compartments, presumably to mimic increased axonal excitability. In our experiments, the muscarininc augmentation of somatic I_{cat} and I_h at resting potentials

would provide a similar tonic depolarization as well as a noninactivating bias current to the dendritic compartment required for the model. However, if ectopic axonal action potentials are critical for γ oscillations as predicted by Traub et al. (2000), then one must also consider how these observed changes in I_h and I_{cat} activity might influence axonal activity. All of our recordings were made from the somata of CA3 pyramidal neurons. However, one would predict that the large somatic depolarization resulting from the increase of I_h or I_{cat} would bring the neuron, including presumably the axon initial segment (and perhaps beyond), closer to the threshold of voltage-dependent sodium channel activation, which presumably underlie ectopic action potential generation. Therefore, a block of I_h (or I_{cat}), if they were solely restricted to somata and dendrites, would be likely to also lower the probability of ectopic action potentials occurring to such an extent that γ oscillations are abolished. It is worthwhile noting that the magnitude of depolarization in response to muscarine application observed in the present set of experiments (e.g., Figure 4A) is approximately three times larger than reported by others (Traub et al., 2000). The reasons for this are at present unclear but may reflect differences due to the use of submerged slices versus slices held in an interface chamber or the use of sharp microelectrodes versus whole-cell patch electrodes.

It is unclear at present where either I_{cat} or I_h conductances are expressed in CA3 pyramidal cell axons. However, one would predict that the presence of either conductance in the axonal compartment would provide the necessary depolarization and increase in excitability, following M1 receptor activation, required for the generation of ectopic action potentials. Indeed, a cAMP-dependent modulation of I_h has been observed in the presynaptic terminals of crayfish neuromuscular junction (Beaumont and Zucker, 2000) and cerebellar basket cells (Southan et al., 2000). In these studies, modulation of I_h enhanced neurotransmitter release properties by altering the excitability of the presynaptic axon (but see Cuttle et al., 2001). Were a similar mechanism to exist in CA3 pyramidal neuron axons, this could lead to the depolarization of presynaptic terminals and the necessary generation of ectopic spiking. However, a presynaptic expression of h channels in cells that express the M1 muscarinic receptor has yet to be described. Moreover, M1 receptors are largely restricted to the somata and dendrites of hippocampal pyramidal neurons (Levey et al., 1995) with little evidence for their presence in axons. Given the somato-dendritic location of the M1 receptor, we would speculate that the modulation of I_h and I_{cat} occurs primarily in the dendritic compartment. How this translates or influences axonal excitability remains to be tested either by in vitro experimentation or by computer modeling.

Thus, many of the model criteria can be fulfilled by the modulation of cation conductances in single CA3 pyramidal neurons described in the present study. Our results show that the tonic depolarization of pyramidal neurons appears to be based mainly on the activation of I_{cat}, but our data also directly demonstrate a role for I_h near the resting membrane potential. The major effect of I_h augmentation, therefore, is likely to be 2-fold: providing a bias current close to the resting membrane potential and altering the pacemaking properties of pyramidal neurons, possibly resulting in change of the firing characteristics of neurons via modulation of the medium duration afterhyperpolarization (Maccaferri et al., 1993; Maccaferri and McBain, 1996). Classically, I_h has been shown to act as a pacemaker current (Maccaferri and McBain, 1996; McCormick and Pape, 1990) to be modulated by neurotransmitters in both neurons (McCormick and Pape, 1990) and cardiac cells (Brown et al., 1979) and to have a role in determining the neuronal resting membrane potential (Halliwell and Adams, 1982; Maccaferri et al., 1993; Magee, 1998). Recent expression studies (Santoro et al., 2000) have shown that CA3 pyramidal neurons express both HCN1 and HCN2 I_h transcripts. In recombinant systems, HCN1 possesses rapid activation kinetics and is presumed to be responsible for the rapid activation kinetic observed in hippocampal pyramidal neurons (Santoro et al., 2000). The time course of I_h activation in the present study ($\tau_{fast} \approx 100$ ms) may enable I_h to play a role in network activity in the γ frequency band. In contrast, in thalamic relay neurons, HCN2 and HCN4 are primarily expressed and impart much slower kinetics ($>$10-fold slower τ activation than hippocampal pyramidal neurons) (Santoro et al., 2000). Consistent with expression of these channels, thalamic oscillations in which I_h plays a gating role are in the much slower delta range (2–4 Hz).

Of particular importance, I_h has recently been shown to modify the integrative properties of hippocampal pyramidal neurons by removing the dependence of temporal integration on the location of synaptic inputs (Magee, 1998, 1999). It is conceivable that such synaptic input normalization leads to enhanced synchrony in network activity. Furthermore, I_h and I_{cat} are major components of the intrinsic current repertoire of most neurons of the hippocampal and neocortical circuitry, and these currents are affected by many different neuromodulators (Soltesz et al., 1991; Colino and Halliwell, 1993; Pape, 1996 Santoro et al., 2000). Therefore, it seems prudent to suggest that I_h and I_{cat} play a fundamental role in γ oscillations, transcending the muscarinic induction paradigm. Future studies will determine the role of these conductances in other models of hippocampal oscillatory activity.

In conclusion, the present findings bring together several lines of research and bridge the gap between cellular currents and neuronal network activity. We show a crucial role for I_h and I_{cat} in muscarine-induced γ oscillations, a network activity that is assumed to be important for higher processes in the CNS (Gray et al., 1989; Singer, 1993). Concomitantly, our data delineate a novel pathway that links the augmentation of I_h and I_{cat} to M1 muscarinic receptor activation.

Experimental Procedures

Generation of M1 Muscarinic Receptor-Deficient Mice

A murine M1 muscarinic receptor clone was isolated from a 129Sv/J mouse genomic library (Genome Systems, Palo Alto, CA) by using a PCR fragment corresponding in sequence to the central portion of the third intracellular loop of the receptor as a probe. The final targeting vector contained two copies of the herpes simplex virus thymidine kinase gene (TK) and a PGK-neomycin resistance cassette (neo), which replaced a 0.28 kb KpnI-SacI genomic fragment

that included the translation start site and the region coding for the first 54 amino acids of the M1 receptor protein (Figure 1A). The targeting vector was linearized and introduced into TC1(129SvEv) embryonic stem (ES) cells (Gomeza et al., 1999b) by electroporation. Clones resistant to G418 and gancyclovir were isolated and screened via Southern blotting for homologous recombination events. Properly targeted ES cell clones were microinjected into C57BL/6J blastocysts to generate male chimeric offspring, which in turn were mated with female CF-1 mice (Charles River) to generate F1 offspring. F1 animals heterozygous for the M1 receptor mutation were then intermated to produce homozygous M1 receptor mutant mice (F2). All experiments were carried out with littermates of the F2 or F3 generation (CF-1 × 129SvEv hybrids).

Radioligand Binding and Immunoprecipitation Studies

Membranes were prepared from different mouse brain tissues essentially as described (Gomeza et al., 1999a, 1999b). For radioligand binding studies, membranes were incubated with a saturating concentration (2 nM) of the muscarinic antagonist, [^{3}H]QNB (Gomeza et al., 1999a). Binding reactions were carried out for 1 hr at room temperature (22°C). Nonspecific binding was determined in the presence of 10 μM atropine. For immunoprecipitation studies, M1–M5 muscarinic receptor-specific rabbit polyclonal antisera were raised against nonconserved regions of the third cytoplasmic loops of the mouse M1–M5 receptor proteins (Gomeza et al., 1999a, 1999b). Membranes preparations were incubated with 2 nM [^{3}H]QNB to label M1–M5 muscarinic receptors. [^{3}H]QNB-labeled receptors were solubilized with 1% digitonin and subjected to immunoprecipitation by M1–M5 receptor-specific antisera as described (Gomeza et al., 1999a, 1999b).

Slice Preparation

Mice were anesthetized by isoflurane volatile inhalation and decapitated according to NIH guidelines. Horizontal hippocampal slices were prepared from 25- to 40-day-old (450 μm thickness, extracellular field recordings) or 13- to 20-day-old (250 μm thickness, patch-clamp recordings) animals of either sex. Slices were maintained at room temperature (22°C) in a submerged holding chamber. For extracellular field recordings, slices were transferred to an interface chamber and maintained at 35°C at the interface between warm, humidified carbogen gas (95% O_2/5% CO_2) and artificial cerebrospinal fluid (ACSF) containing 130 mM NaCl, 3.5 mM KCl, 24 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, and 10 mM glucose. For patch-clamp recordings, slices were transferred to a submerged recording chamber and superfused with ACSF at room temperature.

Electrophysiology

Extracellular field recordings were made in the stratum pyramidale of the hippocampal area CA3 and patch-clamp recordings in pyramidal neurons of area CA3. Cells were voltage-clamped at resting membrane potential unless otherwise stated (-62.9 ± 2.3 mV; $n = 23$). All voltage-clamp experiments were carried out with fast excitatory and inhibitory synaptic transmission as well as voltage-dependent sodium currents blocked by bath application of 20 μM DNQX, 50 μM D-APV, 100 μM picrotoxinin, and 1 μM TTX. Glass microelectrodes (resistance was 3–4 MOhm for both extracellular and patch-clamp recordings) were made from thin-walled borosilicate glass TW150F (WPI, Sarasota, FL) and contained either ACSF (extracellular field recordings) or 42 mM KCl, 60 mM KGlu, 1.5 mM $ATPNa_2$, 0.3 mM $GTPNa_2$, 10 mM HEPES, 2 mM BAPTA, and 3 mM $MgCl_2$ (patch-clamp recordings). Drugs were purchased from Tocris Cookson Ltd. (DNQX, D-APV, and ZD7288) or Sigma (muscarine chloride, linopirdine, TTX, and picrotoxinin). All drugs were diluted directly from frozen stock solutions into the superfusion medium. Patch-clamp and extracellular recordings were made at room temperature using an Axopatch-1D amplifier with the pClamp acquisition software (Axon Instruments, Foster City, CA). Axograph (Axon Instruments) and KaleidaGraph software (Synergy Software) were used for further analyses. Power spectra were obtained over 60 s long recording periods using a Fast Fourier transform algorithm. Data are represented as mean ± SEM. The paired Student's t test was used for statistical analyses.

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