M1 Muscarinic Receptor Modulation of Kir2 Channels Enhances Temporal Summation of Excitatory Synaptic Potentials in Prefrontal Cortex Pyramidal Neurons

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Carr DB, Surmeier DJ. M1 Muscarinic receptor modulation of Kir2 channels enhances temporal summation of excitatory synaptic potentials in prefrontal cortex pyramidal neurons. J Neurophysiol 97: 3432–3438, 2007. First published March 21, 2007; doi:10.1152/jn.00828.2006. The cholinergic innervation of the prefrontal cortex (PFC) plays a pivotal role in regulating executive functions. Muscarinic receptors activated by acetylcholine depolarize pyramidal neurons in the rodent PFC homologue, but the mechanisms mediating this modulation are controversial. To address this question, we studied the responses of layer V rat pre- and infralimbic cortex pyramidal neurons to muscarinic receptor stimulation. Consistent with previous findings, M1 receptor stimulation produced a strong depolarization, leading to tonic firing. Voltage-clamp analysis revealed that M1 activation reduced constitutively active inwardly rectifying (Kir2) K+ channel currents. Blocking protein kinase C activation or depleting intracellular Ca2+ stores did not affect the modulation. However, reversal of the modulation was prevented by the phosphomositide kinase inhibitor, wortmanin, suggesting the modulation was mediated by depletions of membrane phosphatidylinositol-4,5-bisphosphate (PIP2). Reduction of Kir2 channel currents by M1 receptor stimulation significantly increased the temporal summation of excitatory synaptic potentials (EPSPs) evoked by repetitive stimulation of layer I. This action was complemented by M2A receptor mediated presynaptic inhibition of the same terminals. As a consequence of this dual modulation, the responses to a single, isolated afferent volley was reduced, but the response to a high-frequency afferent burst was potentiated.

INTRODUCTION

The release of acetylcholine (ACh) in the cerebral cortex play a critical role in the performance of tasks that require attention (Sarter et al. 2005). ACh levels in the medial prefrontal cortex (PFC) rise during performance of tasks that demand attention (Passetti et al. 2000), and intra-PFC injections of the muscarinic receptor antagonist scopolamine disrupts task performance (Robbins et al. 1998). Recordings from PFC neurons during performance of a sustained visual-attention task revealed that cholinergic deafferentation of this region significantly reduced both baseline firing as well as task-related activity (Gill et al. 2000). In particular, cholinergic deafferentation significantly blunted the enhanced activity of PFC neurons seen in response to the addition of distractors.

It has long been known that stimulation of muscarinic receptors depolarizes cortical pyramidal neurons (Knjjevic 2004). Although some investigators have suggested that the depolarization is mediated by the activation of a nonselective cation current (Fisahn et al. 2002; Haj-Dahmane and Andrade 1996; Klink and Alonso 1997), earlier reports attributed the effect to the closure of a standing potassium (K+) conductance (Benson et al. 1988; Madison et al. 1987). Pyramidal neurons express a broad array of K+ channels but most are not open at the resting membrane potential. One channel that is open, making it a potential candidate, is formed by Kir2 subunits (Stanfield et al. 2002). These inwardly rectifying channels help set the resting membrane potential of PFC pyramidal neurons as well as the integration of excitatory synaptic inputs (Day et al. 2005). The open probability of Kir2 channels is modulated by an interaction with the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP2) (Stanfield et al. 2002). Depletion of membrane PIP2 by activation of membrane phospholipases, such as phospholipase C (PLC), lowers the open probability of Kir2 channels. Because the effects of ACh on pyramidal neurons are largely mediated by M1 muscarinic receptors that stimulate PLC (Benson et al. 1988), it is plausible that closure of Kir2 K+ channels is responsible, at least in part, for the membrane depolarization. The experiments described here were aimed at testing this hypothesis. Our results show that indeed Kir2 K+ channel currents are reduced by M1 receptor stimulation in layer V pyramidal cells of the rodent prefrontal/infralimbic (PL/IL) cortex, resulting in a membrane depolarization. As expected, closure of these channels appears to be dependent on depletion of PIP2 from the membrane. In addition to membrane depolarization, closure of the largely dendritic Kir2 channels dramatically enhances the summation of excitatory synaptic potentials.

METHODS

Male Sprague-Dawley rats, 16–22 day old (median age = 18) were deeply anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) and rapidly decapitated. The brain was quickly removed and immersed in an 0°C artificial cerebrospinal fluid (ACSF) solution containing (in mM) 125 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.25 NaH2PO4, 4 ascorbic acid, and 11 d-glucose. Unless otherwise noted, all reagents were obtained from Sigma (St. Louis, MO). Coronal slices (300 μm) containing the prelimbic and infralimbic cortices (Paxinos and Watson 1998) were cut on a vibrating tissue slicer (Leica) and transferred to a holding chamber where they were incubated in ACSF at 35°C for 30 min. Slices were subsequently

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stored at room temperature until recording. All ACSF solutions were constantly bubbled with 95% O₂–5% CO₂ to maintain pH ~ 7.4. Whole cell current and voltage-clamp recordings were performed using standard techniques. Slices were transferred to a submersion type recording chamber and were constantly perfused (~2 ml/min) with warmed (32 ± 1°C) ACSF. Layer V pyramidal neurons were visualized using either an Olympus BX51 or Zeiss Axioskop 2FS microscope equipped with IR-DIC optics and a Dage-MTI infrared video camera. Glass micropipettes were filled with an internal solution containing (in mM) 130 KMeSO₄, 5 KCl, 2 MgATP, 0.5 Na₂GTP, 5 HEPES, 0.5 CaCl₂, 5 EGTA, and 0.1 spermine, pH 7.3, 270 mosM/l. Spermine was included in the internal solution to maintain the inwardly rectifying character of Kir2 channels (Ficker et al. 1994). The pipette resistance, as measured in the bath, was typically 3.5–4.5 MΩ. Current- and voltage-clamp recordings were obtained using a Multi-clamp 700A or 700B amplifier, a Digidata 1322 A/D converter and pClamp software (Axon Instruments). Records were not corrected for the liquid junction potential, which was estimated to be 6–7 mV. As a consequence, the actual membrane potentials should have been 6–7 mV more negative than those reported here.

Data were analyzed using Clampfit (Axon Instruments) and IgorPro (WaveMetrics) software. Statistical analyses were performed using SPSS (Chicago, IL). In data presented as box plots, the central line represents the median, the edges of the box represent the intraquartile range, and the “whisker lines” show the extent of the overall distribution, excluding points 1.5 times the intraquartile range that are considered outliers and are shown as circles.

All reagents were obtained from Sigma (St. Louis, MO) with the exception of KMeSO₄ (ICN Biochemicals), NaGTP (Roche), TTX (Alomone Labs), CNQX, APV and ZD7288 (Tocris), thapsigargin, and chelrythrine chloride (Calbiochem). All cholinergics, channel blockers and signal transduction reagents were prepared as concentrated stock solutions in distilled water and either added immediately to ACSF at working concentrations or frozen at −20°C until use.

RESULTS

M₁ muscarinic receptor activation produces an inward current and membrane depolarization in PL/IL pyramidal cells

Bath application of the muscarinic cholinergic agonist carbachol (CCh, 10 μM) depolarized pyramidal neurons, leading to repetitive spiking (Fig. 1A). The CCh effect was antagonized by atropine (1 μM, n = 3, data not shown) or the M₁ receptor antagonist pirenzepine (2 μM, n = 4, Fig. 1B) but not by the M₂/M₄ antagonist gallamine (2 μM, n = 4, Fig. 1C). Blockade of AMPA and N-methyl-d-aspartate (NMDA) receptors with kynurenic acid (1.5 mM) or a combination of CNQX (10 μM) and APV (50 μM) did not prevent the CCh induced depolarization and spiking (data not shown), demonstrating that the effect was not synaptically mediated.

When the somatic was voltage clamped near the resting membrane potential (~−70 mV), bath application of CCh evoked a slow inward current (Fig. 1B). Washing CCh out of the slice reversed the change in holding current. As seen in the current-clamp recordings, atropine (n = 3, data not shown) or the M₁ receptor antagonist pirenzepine (2 μM, Fig. 1D, inset, n = 5) blocked the effects of CCh. In contrast, the M₂/M₄ receptor antagonist gallamine (2 μM, Fig. 1D, inset, n = 7) had no effect on the response to CCh.

\[ \text{M}_1 \text{ receptor stimulation reduces inwardly rectifying potassium currents} \]

We have previously shown that blockade of constitutively active inwardly rectifying potassium (Kir2) channels depolarizes PL/IL pyramidal neurons, leading to tonic firing (Day et al. 2005). Given the similarity of the responses to Kir2 channel blockade and M₁ receptor stimulation in these cells, we sought to determine whether M₁ receptors inhibit Kir2 channels. In the presence of TTX (500 nM) and the HCN channel blocker ZD7288 (40–50 μM), somatic hyperpolarizing voltage steps from ~−60 mV evoke a Ba²⁺-sensitive current which is largely carried by Kir2 channels (Fig. 2A) (Day et al. 2005). CCh (10 μM) reduced these currents, particularly at membrane potentials below the K⁺ equilibrium potential. Subtracting currents evoked in the presence of CCh from those evoked in its absence, yielded estimates of the CCh-sensitive current (Fig. 2B). The current-voltage plot of this current was very similar to that of the Ba²⁺-sensitive current (Fig. 2C). Both currents reversed close to the predicted K⁺ reversal potential (E_K) and showed prominent rectification above E_K (Fig. 2C). Similar results were obtained using voltage ramps from −120 to −50 mV (100 ms, Fig. 2D). Scaling the CCh-sensitive current revealed that it rectified more strongly than the Ba²⁺-sensitive current (Fig. 2E), consistent with the notion that CCh prefer-
entially targeted strongly rectifying Kir2 channels. In spite of the rectification, these channels do pass outward current above $E_K$ and reducing their open probability will result in a net inward, depolarizing current.

If the principal action of CCh is to reduce the open probability of $K^+$-selective Kir2 channels, the reversal potential of the CCh-sensitive current should be determined by $E_K$. This was the case, as the reversal potential for the CCh-sensitive current moved in response to elevations in extracellular [K+] as predicted by the Nernst equation (Fig. 3A, $R^2 = 0.86, P < 0.001$). This hypothesis is also consistent with the observation that CCh failed to evoke a significant current in the presence of either Ba$^{2+}$ (200 μM, Fig. 3B, $C n = 6, P < 0.05$, Mann-Whitney) or Cs$^+$ (1 mM, Fig. 3D and $E n = 7, P < 0.05$, Mann-Whitney), two ions that have been shown to block Kir2 channels (Stanfield et al. 2002).

In addition to Kir2 channels, cortical pyramidal neurons express G-protein-activated inwardly rectifying $K^+$ (Kir 3) channels, which are activated by neuromodulators such as serotonin and adenosine (Takigawa and Alzheimer 1999). If these channels were tonically activated by a Gi/o-coupled receptor, membrane depletion of PIP2 by M1 receptors could reduce their open probability (Stanfield et al. 2002). To test this hypothesis, the Kir3 channel blocker tertiapin was used. When applied alone, tertiapin (30 nM) produced little or no change in holding current (Fig. 3G), suggesting that Kir3 channels were not tonically active in our slices. Moreover, tertiapin had no effect on the magnitude of the CCh-sensitive currents (Fig. 3, $F$ and $G; n = 5$), suggesting that modulation of Kir3 channels was not a significant factor in our experiments.

$M_1$ receptors reduce Kir2 channel currents by depleting membrane PIP$_2$

$M_1$ muscarinic receptors are positively coupled to phospholipase C (PLC) by G$_{em}$ proteins (Richardson 1995). To deter-

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**FIG. 3.** $M_1$ receptor stimulation inhibits a Kir2 current in PFC pyramidal cells. A: plot of the reversal potential for the CCh-sensitive current as a function of external [K$^+$]. Points are means ± SE. $n$ for each group is shown in parentheses. Data are fit with the Nernst equation. $B$: currents evoked by a 100-ms voltage ramp from −120 to −50 mV in the presence of TTX and ZD7288. The Kir2 blocker Ba$^{2+}$ (200 μM) produced a significant reduction of the evoked current; however, subsequent addition of CCh (10 μM, gray trace) did not produce any additional effect. $C$: boxplot summary of the CCh-sensitive current measured at −70 mV evoked by this voltage ramp protocol in the presence ($n = 6$) and absence ($n = 6$) of Ba$^{2+}$. $D$: currents evoked by a 800-ms voltage ramp from −120 to −50 mV in the presence of TTX. The Kir2 and HCN blocker Cs$^+$ (1 mM) produced a significant reduction of the evoked current; however, subsequent addition of CCh (10 μM, gray trace) did not produce any additional effect. $E$: boxplot summary of the CCh-sensitive current measured at −70 mV in the presence ($n = 7$) and absence ($n = 6$) of Cs$^+$. $F$: in a representative cell voltage clamped at −70 mV, application of tertiapin (30 nM) produced only a small inward current that did not occlude the inward current elicited by subsequent addition of CCh. $G$: boxplot summary of the inward current induced by CCh ($n = 6$), tertiapin ($n = 5$), or CCh after a 3-min application of tertiapin ($n = 5$).
protein kinase C (PKC), which has been reported to inhibit Kir2 channel currents in heterologous expression systems (Henry et al. 1996; Zhu et al. 1999) and in nucleus basalis neurons (Takano et al. 1995). To determine if PKC was important in PL/IL pyramidal neurons, CCh was applied after a 5- to 10-min incubation in the PKC inhibitor chelerythrine chloride (1 μM). This had no effect on the modulation (Fig. 4, B and D, n = 6), suggesting that the M₁ receptor mediated inhibition of Kir2 channels does not depend on PKC.

In addition to stimulating PKC, activation of PLC also results in the increased production of IP₃, which releases Ca²⁺ from intracellular stores. To test whether the M₁ receptor mediated inhibition of Kir2 currents depended on this linkage, CCh was applied after inhibiting Ca²⁺-ATPase thapsigargin (5 μM), which is known to deplete intracellular Ca²⁺ stores. Thapsigargin had no effect on the response to CCh (Fig. 4, C and D), suggesting that the M₁ receptor modulation of Kir2 channels was not dependent on the release of intracellular Ca²⁺.

Recent studies have shown that Kir2 channel opening is strongly dependent on PIP₂ binding (Stanfield et al. 2002). Depletion of membrane PIP₂ decreases channel open probability, as does M₁ receptor stimulation. Because M₁ receptors activate PLC, which results in the hydrolysis and depletion of PIP₂, this parallel is not surprising. If this is the mechanism by which M₁ receptors close Kir2 channels, then blocking the resynthesis of PIP₂ should prevent reversal of the modulation. Indeed, inhibiting phosphoinositide-3-kinase (PI3K) with wortmannin (40 μM) significantly blunted the reversal of the CCh modulation (Fig. 4, E and F; n = 9; P < 0.01, Mann-Whitney).

M₁ receptor stimulation enhances temporal integration of EPSPs evoked by layer I stimulation

We have previously shown that blockade of Kir2 channels markedly enhances temporal integration of EPSPs (Day et al. 2005). To determine if M₁ receptor stimulation also enhanced temporal summation of EPSPs, we recorded from layer V pyramidal neurons and evoked synaptic potentials with a stimulating electrode placed in layer I. The somatic membrane potential was held near −70 mV to reduce the voltage-dependent effects on measurements of summation. In the absence of CCh, EPSPs evoked by a 40-Hz stimulus train summed sublinearly; the ratio of the amplitude of the fifth to the first EPSP in the train (EPSP5/EPSP1) was typically between 1 and 2 (Fig. 5, A and C). In the presence of CCh (10 μM), the EPSP5/EPSP1 ratio significantly increased to a median value of 4.3 (Fig. 5, A and C; n = 5, P < 0.05, Wilcoxon signed-ranks test). In addition, CCh significantly reduced the amplitude of the first EPSP in the train (Fig. 5, A and B, inset; n = 5, P < 0.05, Wilcoxon signed-ranks test). This observation is consistent with previous reports of M₁/M₄ muscarinic receptors located on glutamatergic terminals (Mrzljak et al. 1993) which act to reduce evoked glutamate release (Kimura and Baughman 1997; Levy et al. 2006). To determine if the CCh-induced enhancement of summation was due to an action on these presynaptic receptors (Brenowitz and Trussell 2001), the experiments were repeated in the presence of the M₁/M₄ antagonist, gallamine (2 μM). In this situation, the amplitude of the first EPSP in the train was not reduced by CCh (Fig. 5B, inset,
DISCUSSION

The studies reported here argue that M₁ muscarinic receptor activation results in closure of constitutively active, inwardly rectifying Kir2 K⁺ channels in layer V PL/IL pyramidal neurons. The closure of Kir2 channels depolarizes PL/IL neurons and leads to tonic spiking. This modulation also enhances the temporal integration of dendritic excitatory synaptic inputs from superficial cortical layers, complementing the presynaptic inhibition of glutamate release mediated by M₂4 muscarinic receptors. These studies suggest that a primary consequence of ACh release on PL/IL pyramidal neurons is the attenuation of isolated single EPSPs and the potentiation of EPSPs generated by spatially and temporally coherent inputs.

M₁ receptors target Kir2 channels in PL/IL pyramidal cells

In agreement with previous anatomical and physiological studies (Krnjevic 2004), the effects of CCh on pyramidal neuron excitability were mediated by largely by pirenzepine-sensitive M₁ muscarinic receptors. The experiments presented here suggest that activation of these receptors led to a reduction in inwardly rectifying K⁺ currents that were attributable to flux through Kir2 channels that are known to be robustly expressed by these neurons (Day et al. 2005). The involvement of Kir2 channels is supported by four observations. First, the CCh-sensitive current was inwardly rectifying, having a current-voltage profile strongly resembling that of Kir2 channels in other preparations (Stanfield et al. 2002). Second, channels targeted for modulation were K⁺ selective as flux through them reversed near Eₖ. Third, the CCh-sensitive current was blocked by Cs⁺ or Ba²⁺, both of which block Kir2 channels in the concentration range used. Last, the M₁ receptor modulation was not altered by disruption of PKC or intracellular Ca²⁺ signaling (Haj-Dahmane and Andrade 1998) but rather PIP₂ metabolism. Binding of PIP₂ to the C-terminus of Kir2 channel subunits is necessary for channel opening (Stanfield et al. 2002). Our observations are consistent with a growing literature showing M₁ muscarinic receptor stimulation of PLC leads to depletion of membrane PIP₂ and closure of Kir2 channels (Chuang et al. 1997; Du et al. 2004). Interestingly, the sensitivity of Kir2 channels to depletion of PIP₂ and modulation by G-protein-coupled receptor (GPCR) signaling cascades through PLC is dependent on the subunit composition of the channels. There are four Kir2 subunits that have been cloned (Kir2.1–4). Channels composed of Kir2.3 subunits, which are prominently expressed in PL/IL pyramidal neurons (Day et al. 2005), are very sensitive to alterations in membrane PIP₂ content, and activation of PLC linked GPCRs, like the M₁ muscarinic receptor (Du et al. 2004).

M₁ receptors modulation of Kir2 K⁺ channels is likely to be part of a coordinated response

Our results are consistent with previous observations from neurons in the hippocampus, cortex, and striatum showing that muscarinic receptor stimulation reduces opening of K⁺ channels with properties resembling those of Kir2 channels (Hsu et al. 1996; Krnjevic 2004; Uchimura and North 1990). However, others have attributed the depolarizing actions of muscarinic receptor stimulation in pyramidal neurons to the opening of a mixed cation channel (Fisahn et al. 2002; Haj-Dahmane and Andrade 1998).
ACh, Kir2 channels, and cortical state transitions

In vivo intracellular recordings of cortical pyramidal neurons in either anesthetized or sleeping animals exhibit large-amplitude, rhythm fluctuations in membrane potential characterized by periods of depolarization (approximately 55 mV) and irregular spike firing interspersed with large-amplitude (10–20 mV), long-lasting (~500 ms) hyperpolarizations. (Metherate et al. 1992; Steriade et al. 2001). The transition from sleep to active waking is accompanied by the loss of these long-lasting, large amplitude hyperpolarizing periods (Steriade et al. 2001). The standing outward current generated by Kir2 channels should help to stabilize these hyperpolarized epochs. There are several pieces of evidence that suggest the shift to tonic depolarization during wakefulness is due to Ach-mediated suppression of these channels. As animals transition from sleep to active waking, cortical ACh levels increase (Marrosu et al. 1995) and in parallel the input resistance of cortical neurons increases (Steriade et al. 2001), consistent with a closure of Kir2 channels during wakefulness. In anesthetized animals, stimulation of the nucleus basalis (NB), the principal source of cortical ACh, blocks these large amplitude hyperpolarizations (Metherate et al. 1992). This effect of NB stimulation is prevented by iontophoresis of atropine (Metherate et al. 1992) and mimicked by intracellular perfusion with the Kir2 channel blocker cesium (Metherate and Ashe 1993).

Cholinergic modulation of Kir2 channels enhances temporal summation of EPSPs

Previous work has shown that Kir2.2 and Kir2.3 channels are expressed in the dendrites of PFC pyramidal neurons where they influence the temporal integration of distally generated EPSPs (Day et al. 2005). Here, where cells were manually “clamped” near their resting membrane potential, M1-mediated inhibition of Kir2 channels produced a significant enhancement in the temporal summation of evoked EPSPs. This enhancement is largely attributable to the elevation in dendritic input impedance after Kir2 channel closure (Day et al. 2005). In the absence of a somatic voltage clamp, inhibition of Kir2 channels also will depolarize the dendritic membrane and deactivate HCN channels (Day et al. 2005). The deactivation of HCN channels should work in concert with the closure of Kir2 channels to enhance the temporal summation of EPSPs (Day et al. 2005; Magee 2000).

The cortical effects of ACh are not limited to modulation of postsynaptic Kir2 channels in pyramidal neurons. CCh also significantly reduced the amplitude of the first EPSP in the stimulus train. As closure of Kir2 channels should increase the amplitude of the first EPSP, this modulation is likely due to a presynaptic action. Previous work has shown that presynaptically located M2/4 muscarinic receptors (Mrzljak et al. 1993) reduce glutamate release at intracortical terminals (Kimura and Baughman 1997; Levy et al. 2006). In agreement with these observations, the M2/4 antagonist gallamine blocked the CCh-induced suppression of the initial EPSP, arguing that this component of the response was presynaptically mediated. This distribution of effects differs from that found in hippocampal CA1 pyramidal neurons where postsynaptic M2/4 muscarinic receptors activate Kir3 channels, reducing the amplitude of the response to exogenous glutamate application (Seeger and Alzheimer 2001). The absence of a CCh-evoked current in the presence of the M1 antagonist, pirenzepine (Fig. 1D, inset), argues against the presence of similar mechanisms in PF/IF layer V pyramidal neurons. In fact, the presynaptic- and postsynaptic effects of ACh in PF/IF pyramidal neurons complement one another. By reducing release probability, presynaptic inhibition increases the fidelity of the synaptic response to repetitive stimulation (Brenowitz and Trussell 2001). As shown here, the postsynaptic reduction in Kir2 channel currents enhances the temporal summation EPSPs. The combination of these effects led to larger enhancement in the EPSP5/ EPSP1 ratio than seen with just the postsynaptic M1 receptor modulation alone (Fig. 5C). Thus ACh reduced the postsynaptic consequences of a single, isolated presynaptic event but enhanced the response to a temporally coherent burst of synaptic activity. These findings suggest that elevations in PFC ACh levels during the performance of working-memory tasks, serve to enhance the response to salient, sustained stimuli while suppressing the response to weaker, transient distracting events.

GRANTS

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