Muscarnic M<sub>2</sub> and M<sub>1</sub> Receptors Reduce GABA Release by Ca<sup>2+</sup> Channel Modulation Through Activation of PI<sub>3</sub>K/Ca<sup>2+</sup>-Independent and PLC/Ca<sup>2+</sup>-Dependent PKC

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INTRODUCTION

Acetylcholine is released by a nonsynaptic network of axon terminals originating from the nucleus basalis of Meynert (NB) into the neocortex where it regulates attention (Passetti et al. 2000; Voytko et al. 1994), cortical rhythms (Buh et al. 1998; Podols’kii et al. 2000), and plasticity (Kilgard and Merzenich 1998). The attentional control of cortical acetylcholine release is supposed to be regulated by a bi-directional prefrontal cortex (PFC)–NB circuit, whose activation, in turn, induces acetylcholine release on more posterior cortical areas (Sarter et al. 2005a). Impairment of the regulation of cortical cholinergic function is associated with, and at least in part responsible for, the occurrence of Alzheimer disease (cholinergic deficit) and schizophrenia (cholinergic excess, Sarter et al. 2005a,b).

Activation of the virtually ubiquitous muscarinic acetylcholine receptors (Mash and Potter 1986) on GABA–containing (GABAergic) interneurons controls many functions including burst synchronization (Kondo and Kawaguchi 2001), information flow across cortical layers (Yang et al. 1998), sensory receptive fields (Restuccia et al. 2003), and γ-oscillations generation (Fisahn et al. 1998), prompting at GABAergic interneurons as an exquisitely sensitive target for cholinergic control of neocortical function.

The auditory cortex is subject to strong cholinergic modulation (Arakimas et al. 1997; Hsieh et al. 2000; Kilgard and Merzenich 1998; McKenna et al. 1988; Metherate and Ashe 1991), contributing to processing and storage of auditory information with a variety of cellular mechanisms. Previous studies reported that activation of muscarinic receptors, along with other cellular actions, decreases GABAergic synaptic currents in the auditory cortex (Metherate and Ashe 1995). Limited information is available on the cellular mechanisms and physiological significance of the cholinergic decrease of the GABAergic synaptic activity, representing a crucial component of the physiological and pathological increase in excitability of the auditory neocortex.

In this work, we quantified the cellular and pharmacological characteristics of the muscarinic modulation of GABAergic currents within the first cortico-cortical relay, constituted by the cells of layer II/III of the auditory cortex. We found that activation of muscarinic receptors reduces the release of GABA in the auditory cortex to approximately one third by inhibiting presynaptic voltage-gated Ca<sup>2+</sup> channels mainly through two pathways: the PI<sub>3</sub>K/Ca<sup>2+</sup>-independent PKC-mediated pathway and the PLC/Ca<sup>2+</sup>-independent PKC-mediated pathway. The resulting decrease in GABAergic function has the potential to shift between cortical states by drastically changing neocortical excitability using previously unknown synaptic mechanisms.

METHODS

Preparation

We used an auditory cortex slice preparation similar to one previously described (Atzori et al. 2001). Twenty-three to 40-day-old...
Sprague-Dawley rats (Charles River, Wilmington, MA) were anesthetized with isoﬂurane (Baxter, Round Lake, IL) and killed according to the National Institutes of Health guidelines (UTD IACUC number 04-04), and their brains were sliced with a vibratome (VT1000, Leica) in a refrigerated solution (0–4°C) containing (mM) 130 NaCl, 3.5 KCl, 10 glucose, 24 NaHCO₃, 1.25 NaH₂PO₄, 1.5 CaCl₂, and 1.5 MgCl₂, saturated with a mixture of 95% O₂-5% CO₂ (ACSF). Two hundred seventy-micrometer-thick coronal slices from the most caudal fourth of the brain were retained after removing the occipital convexity and subsequently incubated in ACSF at 32°C before being placed in the recording chamber. The recording area was selected dorsally to the sylvian sulcus corresponding to the auditory cortex (Rutkowski et al. 2003). The recording solution also contained 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM) and kynurenic acid (2 mM) for blocking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)- and N-methyl-d-aspartate receptor (NMDAR)-mediated currents, respectively.

**Drugs and solutions**

All drugs were purchased from Sigma (St. Louis, MO), TOCRIS (Ellisville, MO), Peptides International (Louisville, KY), or Alomone Laboratories (Jerusalem, Israel). The muscarinic toxin MT1 was a generous gift from Alomone Laboratories. In some experiments, pulses of the GABA_A agonist muscimol (100 μM) were applied at 100–200 μm from the recording areas, once every 30 s. A stock solution of muscimol was diluted 10-fold in ACSF before being back-filled to a glass pipette similar to the one used for recording. Muscimol application was performed using a pressure system (pico-spritzer, General Valve, Fairfield, NJ) through a glass pipette (≈25 psi, 3–12 ms). Stock solutions of all drugs were prepared in water except for U73122, AF-DX 116, and bisindolylmaleimide, whose stock solutions were prepared in dimethylsulfoxide (final concentration, 0.1%). For nonaquesous solutions, the final concentration of the solvent was added to the recording control solution. Drugs were bath-applied into the recording chamber except for U73122, pertussis toxin (PTX), wortmannin, LY-294002, bisindolylmaleimide, and Go6976, which were added to the incubation chamber as a pretreatment as specified in the text. PTX was activated according to Kaslow et al. (1987). After recording an initial baseline for 7–10 min, drugs were bath-applied for 5 min or longer, until reaching a stable condition (see **Statistical analysis**).

**Electrophysiology**

Slices were placed in an immersion chamber, where cells with a prominent apical dendrite, suggestive of pyramidal morphology, were visually selected using a BX 51 (Olympus) with an infrared violet Nissl counterstain was used to identify the cortical layers. The recording solution also contained a standard ABC kit (Vector Labs, Burlingame, CA). A light cresyl staining was processed using diaminobenzidine as chromogen, using a standard ABC kit (Vector Labs, Burlingame, CA). A light cresyl violet Nissl counterstain was used to identify the cortical layers.

**Immunofluorescence**

Brains for immunohistochemistry were extracted from three rats previously anesthetized using 30% isoﬂurane and perfused transcardially with 0.1 M PBS, followed by 4% paraformaldehyde fixative. Once removed from the skull, brains were postfixed overnight at 4°C in the same solution and stored at the same temperature in cryoprotective solution (20% glycerol) until sectioning. Forty-micrometer-thick sections were cut on a microtome (Micron HM 430) and stored at 4°C in 0.1 M PBS with 0.001% NaN₃. Free-floating sections were washed in 0.1 M PBS and incubated for 1 h at room temperature (RT) in blocking buffer [0.1 M PBS, 3% normal goat serum (NGS), and 0.3% Triton X-100]. Primary antibodies were diluted in dilution buffer containing 0.1 M PBS, 1% NGS, and 0.3% Tween-20. Sections were incubated with monoclonal rat anti-M2AchR (1:50; MAB367, Chemicon) and either rabbit anti-parvalbumin (1:6,000; AB9312, Chemicon), mouse anti-somatostatin (1:10; V1169, Biomeda), or rabbit anti-calbindin (1:2,500; AB1778, Chemicon) at 4°C for 48 h on a shaker. After primary incubation, sections were washed 5 times for 10 min with 0.1 M PBS. The sections were incubated for 2 h at RT with the respective secondary antibodies (Cy-5 Goat Anti-Rat and Cy-2 Goat Anti-Rabbit or Cy-2 Goat Anti-Mouse, Jackson Immunoresearch) diluted 1:200 in 0.1 M PBS. After secondary incubation, sections were washed 5 times for 10 min with 0.1 M PBS and mounted on glass slides with DPX (Fluka 44581) and observed using a confocal microscope.

**Statistical analysis**

We defined a statistically stable period as a time interval (3–5 min) along which the IPSC mean amplitude measured during any 1-min assessment did not vary according to an unpaired Student’s t-test. Means ± SE are reported. Pair ratio (P) was calculated as the mean of the second response divided by the mean of the first response, according to Kim and Alger (2001).

The effects of drug application on the IPSC amplitude changes are reported as R (reduction) = 100 × (1 – A_treat/A_ctrl), where A_treat and A_ctrl were, respectively, the mean IPSC amplitude in treatment or in control (R = 0 corresponded to no change, whereas R = 100 corresponded to total block). Drug effects were assessed by measuring and comparing the different parameters (R, IPSC mean amplitude, PPR, and others) between baseline (control) versus treatment, with paired Student’s t-test. ANOVA unpaired Student’s t-tests were used for comparisons between different groups of cells, and the Wilcoxon test was used for comparing between PPRs. Data were reported as different only if P < 0.05, unless indicated otherwise. Single asterisks (*) indicate P < 0.05, double asterisks (**) indicate P < 0.01. Quantal analysis was performed to identify the locus of effect of drugs affecting postsynaptic current amplitudes. We used a CV analysis similar to the one reported by Faber and Korn (1991) and by Zucker and Regehr (2002) to determine the synaptic locus, and by Clements and Silver (2000) to calculate eIPSC quantum amplitude.
RESULTS

Pharmacologically isolated GABAergic currents were measured in neurons voltage-clamped at $V_h = -60$ mV. Input resistance was $219 \pm 24 \text{ M} \Omega (n = 16)$. eIPSCs were blocked by bicuculline (10 μM, $n = 6$), indicative of their GABAergic origin (Fig. 1A; time-course in Fig. 1B). Biocytin staining showed that the majority of the recorded cells were spiny neurons displaying either a stereotypical pyramidal morphology with extensive basal dendrite and an apical dendrite portraying a characteristic fan-like expansion in the superficial layers (Fig. 1C) or a more radial symmetric dendritic arborization (Fig. 1D).

Effect of oxotremorine on GABA_A_R-mediated currents

To study the effect of the activation of muscarinic receptors on the inhibitory synaptic signals, we bath-applied the prototypical muscarinic agonists muscarine (1 μM) or oxotremorine (10 μM) after recording a stable baseline response for 7–10 min. We defined the reduction as $R = 100 \times (1 - A_{\text{treat}}/A_{\text{ctrl}})$ ($R = 100$ equals total block). Application of either drug greatly decreased the mean eIPSC amplitude ($R = 57 \pm 7\%$ decrease in muscarine, 6/7 cells; Fig. 2A) without changing the input resistance $>10\%$. Muscarine and oxotremorine also increased paired pulse ratio (PPR), defined as the ratio between the mean of the second to the mean of the first eIPSCs amplitudes (Kim and Alger 2001) (PPR: $1.01 \pm 0.08$ in control vs. $1.33 \pm 0.06$ in muscarine, $n = 6$, $P < 0.02$; Fig. 2C; PPR: $0.9 \pm 0.06$ in control vs. $1.25 \pm 0.9$ in oxotremorine; Fig. 2D; $n = 14$, $P < 0.01$, Wilcoxon’s t-test). The eIPSC amplitude decrease was prevented by previous application of the muscarinic blocker atropine (3 μM, $R = 5 \pm 3\%$, $n = 3$; Fig. 2E), confirming the muscarinic nature of the depression. Mean values of the synaptic amplitude and PPR are reported in Fig. 2, F and G.

We did not detect any effect of muscarinic agonists on the eIPSC kinetics (rise-time $= 2.2 \pm 0.2$ ms in control vs. $2.3 \pm 0.2$ ms in oxotremorine, not significant; decay time was $35 \pm 1.5$ ms in control vs. $34 \pm 2$ ms in oxotremore, not significant).

Synaptic locus of the muscarinic-induced IPSC depression

A decrease in eIPSC amplitude accompanied by an increase in PPR might reflect a change in neurotransmitter release probability (Baldelli et al. 2005). To test the hypoth-

![FIG. 1. GABAergic currents in supra-granular auditory cortex layers. A: synaptic currents were evoked by electrical stimulation at $<300$ μm from the recording site with a pair pulse protocol (interpulse interval ($= 50$ ms) at holding potential $V_h = -60$ mV in the presence of the glutamate receptor blockers DNQX (10 μM) and kynurenate (2 mM). Traces are averages of 20–30 adjacent traces in control, after bicuculline application, and after bicuculline washout. B: time-course of effect of bicuculline. Evoked inhibitory postsynaptic current (eIPSC) amplitude of the 1st response is reported vs. time (stimulation frequency, 0.1 Hz). Responses are reversibly blocked by bicuculline, proving their GABAergic origin. C: camera lucida drawing of a typical cortical spiny cell with basal dendrites and neuropil expansion of its apical dendrite. Calibration bar 100 μm. Symbols (a) show label axonal processes. D: supra-granular cell with more symmetric morphology and radial dendritic arborization.](http://jn.physiology.org/))
esis of a presynaptic involvement, we systematically performed the analysis of the eIPSC CV, which showed an increase in CV after oxotremorine application (0.23 ± 0.025 in control vs. 0.47 ± 0.040 in oxotremorine, P < 0.0001, Student t-test), as shown in the example in Fig. 3A. Similar to the muscarinic amplitude change, the increase in CV (shown in Fig. 3B for each individual recording) is in large part reversible. In Fig. 3C, CV² analysis indicates a presynaptic locus (Faber and Korn 1991). Quantal amplitude (Q) derived from quantal analysis (Clements and Silver 2000)
was not changed by oxotremorine \((Q = 10 \pm 1 \text{ pA in control vs. } Q = 11 \pm 2 \text{ pA in oxotremorine, not significant, data not shown})\).

As a second assay, we used brief pressure applications of the GABA\(_A\) agonist muscimol \((100 \text{ } \mu\text{M})\) to test the effect of oxotremorine on the chemically evoked postsynaptic currents (cIPSC). cIPSC resulted in prolonged inward currents decaying in several seconds, greatly outlasting drug applications (lasting only a few milliseconds, see Methods). Oxotremorine failed to alter cIPSC amplitude (representative example in Fig. 4A; mean in Fig. 4B; \(R = -5 \pm 4\%, n = 10, \text{not significant}\)).

We tested the hypothesis that the activation of muscarinic receptors was directly affecting presynaptic release mechanisms. To do so we measured the effect of oxotremorine on the amplitude and frequency of miniature IPSCs (mIPSCs) in the presence of the Na\(^+\)-channel blocker TTX \((0.5 \text{ } \mu\text{M})\). Neither the frequency nor the amplitude of mIPSC in TTX was altered by application of the muscarinic agonist (representative traces and cumulative histograms of amplitude and frequency are shown in Fig. 4, C–E, respectively). Mean eIPSC amplitude was \(14.5 \pm 0.2 \text{ pA in oxotremorine versus } 14 \pm 0.2 \text{ in control (Fig. 4F; same sample, not significant). Mean frequency was } f = 1.10 \pm 0.30 \text{ Hz in oxotremorine versus } 1.07 \pm 0.20 \text{ Hz in control (Fig. 4G; } n = 11, \text{not significant).}\)

The invariance of the quantal amplitude, rise- and decay-time, muscimol-evoked currents, and mIPSC amplitudes after oxotremorine application, together with the change in eIPSC PPR, indicated that the eIPSC current depression is not postsynaptic. The failure of oxotremorine to decrease mIPSC frequency indicated that voltage-gated channels involved in the release of GABA at the axon terminal were possible targets of the muscarinic modulation.

![Synaptic locus of the muscarinic-induced IPSC depression.](image)
Calcium channels are the target of muscarinic receptor activation

We tested the possibility that the activation of muscarinic receptors induced the decrease in GABA release by targeting axonal calcium channels by first determining which type of Ca\(^{2+}\) channels were responsible for the release of GABA. Application of the selective N-type Ca\(^{2+}\) channel blocker \(\omega\)-conotoxin GVIA (\(\omega\)-CgTxGVIA, 1 \(\mu\)M) or of the P/Q-type Ca\(^{2+}\) channel blocker \(\omega\)-agatoxin TK (\(\omega\)-AgaTK, 200 nM) depressed eIPSC amplitude by 44.5 ± 3 (n = 11) and 78.3 ± 7% (n = 8), respectively. Application of either Ca\(^{2+}\) channel blocker changed PPR (PPR = 0.99 ± 0.09 in control vs. 1.2 ± 0.07 in \(\omega\)-CgTx GVIA, \(P < 0.03\), Wilcoxon’s t-test; PPR = 0.73 ± 0.08 in control vs. 1.1 ± 0.1 in \(\omega\)-agatoxin TK, \(P < 0.02\), Wilcoxon’s t-test) confirmed the presynaptic nature of the effect. Examples of time-course and traces are reported in Fig. 5A for \(\omega\)-CgTxGVIA (PPR for each individual cell in Fig. 5B) and in Fig. 5C for \(\omega\)-AgaTK (PPR for individual cells in Fig. 5D). The mean and PPR for the whole sample is shown in Fig. 5, E and F, respectively. These results indicate that the relative contribution of P/Q- versus N-type Ca\(^{2+}\) channels to GABA release in the cortex is approximately in the ratio 2:1. \(\omega\)-CgTxGVIA did not affect the eIPSC amplitude in 3/14 recordings, suggestive of an axonal fiber population void of N-type channels.

We next tested the effectiveness of oxotremorine in reducing eIPSC in the presence of \(\omega\)-CgTxGVIA or \(\omega\)-AgaTK. In the presence of either toxin, oxotremorine was much less effective in depressing the eIPSC, as shown in the representative time-courses in Fig. 5, G and I, indicating that both N- and P/Q-type Ca\(^{2+}\) channels are involved in the muscarinic-induced depression of the eIPSC amplitude (\(R = 33 ± 0.08\) in \(\omega\)-CgTx GVIA alone vs. 0.7% vs. \(\omega\)-Aga TK plus oxotremorine, \(P = 0.05\), ANOVA with post hoc Tuckey’s test; Fig. 5H; in presence of \(\omega\)-Aga TK alone PPR = 1.01 ± 0.1 vs. \(\omega\)-Aga TK plus oxotremorine, \(P = 0.1\), \(P < 0.05\) Wilcoxon’s t-test; Fig. 5J), confirming again its presynaptic effect. Summary of the action of oxotremorine in the presence of the Ca\(^{2+}\) blockers on PPR is shown in Fig. 5L.

Muscarinic receptors responsible for the depression of GABA release

Because activation of muscarinic M_1Rs reduces GABA release in the visual cortex (Kimura and Baughman 1997), we tested the hypothesis that the same receptor type was respon-
sible for the depression of the GABA signal in the auditory cortex. To test this hypothesis, we measured the effect of the selective M1R agonist MT1 (100 nM) on eIPSC amplitude. MT1 decreased eIPSC amplitude albeit to a lesser extent (25 ± 7%; example of time-course in Fig. 6A; n = 8) with respect to the depression elicited by either oxotremorine (R = 69%) or muscarine (R = 57%, P < 0.04, ANOVA with post hoc Tukey’s test). Consistently with the latter result, the presence of the specific blocker for M1Rs MT7 (20 nM) had a statistically significant but modest effect in preventing oxotremorine from decreasing eIPSC amplitude (R = 44 ± 3%, n = 7, P < 0.03, ANOVA with post hoc Tukey’s test; Fig. 6B). On the
contrary, the presence of the M₃R blockers methoctramine (5 μM) or AF-DX 116 (1 μM) successfully prevented eIPSC depression (R = 14 ± 5%, n = 7 and 18 ± 5%, n = 7, respectively; Fig. 6, C and D; P < 0.01, ANOVA with post hoc Tukey’s test). The M₃R-blocker MT3 (50 nM) or the M₃R-blocker 4-DAMP (100 nM) failed to block the depressant action of oxotremorine on the eIPSC (R = 54 ± 7 and 58 ± 4.5%, P > 0.05 for both, not significant, ANOVA with post hoc Tukey’s test; Fig. 6, E and F). In summary, as shown in Fig. 6G, displaying mean ± SE from the experiments reported above, M₁Rs and M₂Rs seem to be responsible for about one third and two thirds, respectively, of the depression of GABA.

Co-localization of M₂Rs and interneuronal markers

Although the presence of M₁Rs has been documented in both pyramidal cells and GABAAergic neurons (Kimura and Baughman 1997; Mash and Potter 1986; Perez-Rosello et al. 2005), the role and localization of cortical M₂Rs has been less studied. For this reason, we studied the possible co-localization of M₂Rs with the interneuronal marker, parvalbumin (PV), calbindin (CB), or somatostatin (SOM). Commercial antibodies were used (see METHODS) to identify the presence of the corresponding antigens with fluorescent methods. We first performed a series of control experiments with 1) only one primary for the interneuronal marker or M₂Rs plus both secondary antibodies, 2) one primary and the nonmatched secondary antibody, 3) both secondary but no primary antibodies, or 4) single labeling either primary + matched secondary. PV- and CB-positive cells were detected in a relatively large neuronal population spanning through the six cortical layers (Fig. 7), including round-shaped cell bodies, whereas the density of SOM-positive cells was much lower than that of PV- or CB-positive cells (~10 times lower, data not shown). M₂R-positive cells were also detected throughout the cortical layers and corresponded to a relatively large neuronal population with either pyramidal-like or round cells bodies. At the dilutions indicated (see METHODS), all control experiments gave results consistent with the specificity of the antibodies. Double labeling experiments in auditory cortex sections from three animals displayed that approximately one half (50.4%) of the PV-positive cells resulted positive for M₂Rs, whereas 92.9% of the CB-positive cells were also M₂R-positive (Fig. 7, D–F). SOM-positive cells as well displayed a high degree of co-localization with M₂R-positive cells (70.4%). All these data corroborate our hypothesis that M₂Rs are present in GABAAergic neurons.

Both M₁Rs and M₂Rs affect the ω-CgTxGVIA–insensitive component of GABA release

We concluded this series of experiments by determining the contribution of M₁Rs and M₂Rs to the ω-CgTxGVIA–insensitive component of GABA release (presumably P/Q type channel-dependent). We found that, in the presence of ω-CgTxGVIA plus the M₁R antagonist MT7, oxotremorine did not block eIPSCs (R = 8 ± 3%; representative time-course in Fig. 8A), whereas in ω-CgTxGVIA plus the M₂R antagonist AF-DX 116, the block was R = 23 ± 3% (representative time-course in Fig. 8B), suggesting that, although M₂Rs modulate mainly N-type channels, M₁Rs modulate P/Q-type channels (summary of the mean reduction ±SE in Fig. 8C).

PKC and Pl₁K mediate most of the muscarinic GABAergic depression

To test whether M₂Rs activated an intracellular second-messenger cascade associated with the Gₛₒ–pertussis-sensitive family, we incubated our slices in PTX (50 μg/ml), which blocks the effect of Gₛₒ proteins. Although 8- to 12-h slice incubation was not sufficient to prevent muscarinic block (R = 61 ± 5%, n = 8, P > 0.05, ANOVA with post hoc Tukey’s test), 14- to 18-h incubation did decrease significantly the muscarinic eIPSC reduction (R = 39 ± 8%, n = 6, P < 0.05, ANOVA with post hoc Tukey’s test). The incomplete block of the muscarinic effect by PTX might be caused by the slow action of the toxin in the tissue.

M₁Rs, M₃Rs, and M₅Rs are supposed to exert their action through PLC. To test whether PLC activation was involved in the muscarinic eIPSC amplitude reduction, we incubated the slices in the presence of the PLC blocker U73122 (10 μM), obtaining only a slight but statistically significant decrease in oxotremorine-induced eIPSC amplitude depression (R = 43 ± 3% in U73122, n = 13 vs. 69 ± 6% in control, P < 0.02, ANOVA with post hoc Tukey’s test; time-course and representative traces in Fig. 9A). In the presence of U73122, oxotremorine application still increased PPR (1.60 ± 0.10 in oxotremorine vs. 1.31 ± 0.05 in control, P < 0.05). These data suggest that only about one third of the muscarinic modulation was mediated by activation of M₁Rs, indicating that PLC is responsible for only a minor component of the muscarinic-induced eIPSC depression.

We tested the involvement of the Pl₁K in the eIPSC depression by incubating slices for 2 h or longer in the presence of a specific blocker of the Pl₁K, wortmannin (200 nM) or LY210004 (1 μM). Slice incubation with either blocker almost completely prevented oxotremorine-induced eIPSC amplitude depression (R = 15 ± 7% in wortmannin, n = 8, P < 0.01, ANOVA with post hoc Tukey’s test; R = 20 ± 3% in LY210004, n = 6, P < 0.01, ANOVA with post hoc Tukey’s test; Fig. 9, B and C). Application of wortmannin or LY210004 depressed but did not completely prevent the increase in PPR (1.24 ± 0.07 in control vs. 1.42 ± 0.06 in wortmannin, P < 0.05; 1.10 ± 0.05 in control vs. 1.23 ± 0.07, P < 0.05 in LY210004, P < 0.05), suggesting that the nature of the remaining component of the muscarinic depression was still presynaptic.

Because PLC and Pl₁K use different types of PKC downstream of their metabolic cascades (Callaghan et al. 2004; Krieg et al. 2002; Oldenburg et al. 2002; Qin et al. 2003), we wanted to study the nature of the possible contribution of different types of PKC in muscarinic eIPSC depression. To do so, we tested the effect of oxotremorine on slices preincubated with either the nonspecific PKC blocker bisindolylmaleimide (1 μM) or the specific blocker of the Ca²⁺-dependent PKC Go6976. Bisindolylmaleimide incubation completely blocked the muscarinic effect (Fig. 9D; representative time course, R = 9 ± 4%, n = 8, P < 0.01, ANOVA with post hoc Tukey’s test), whereas Go6976 pretreatment produced results similar to those obtained in the presence of MT-7 and U73122 (R = 44.5 ± 2%, P < 0.01 and P < 0.05, ANOVA with post hoc Tukey’s test, n = 7; Fig. 9E), suggesting that Go6976 only occcludes the effect produced by M₂Rs. This hypothesis was confirmed by testing the effect of oxotremorine in the presence of Go6976 + the
M2R blocker AF-DX 116. The simultaneous presence of Go6976 and AF-DX 116 completely blocked eIPSC depression (\( R = 5 \pm 2\%, n = 8, P < 0.01, \) ANOVA with post hoc Tukey’s test; Fig. 9F). The results are summarized in the bar graph in Fig. 9G.

The previous results show that 1) the whole muscarinic eIPSC depression is PKC-dependent; and 2) both PKC isoforms, \( \text{Ca}^{2+}\text{-dependent} \) and \( \text{Ca}^{2+}\text{-independent} \), are responsible for the depression, but M2Rs only activate the latter one (\( \text{Ca}^{2+}\text{-independent} \)), whereas M1Rs only activate a \( \text{Ca}^{2+}\text{-dependent} \) PKC.

**DISCUSSION**

Pharmacologically isolated bicuculline-sensitive postsynaptic currents were inhibited in an atropine-sensitive manner by the prototypical muscarinic agonists muscarine and oxotremorine, indicating that GABAergic synaptic currents were blocked by the activation of acetylcholine muscarinic receptors.

**Synaptic localization of the muscarinic effect**

Several lines of evidence allowed us to trace the synaptic origin of the muscarinic-induced inhibition of GABAergic signal. First, the increase in PPR, CV, and CV² analysis, and the invariance in quantal size are all suggestive of a presynaptic origin. Second, mean mIPSCs rise-time, decay-time, and amplitude did not change after oxotremorine application. Third, muscimol-induced currents were also not sensitive to oxotremorine applications. Although we cannot exclude that the GABAergic terminals generating mIPSCs belonged to a dif-
A muscarinic-induced, PKC- and PI3K-dependent postsynaptic increase in the amplitude of GABA_{A}R-mediated currents has been recently described in the prefrontal cortex (PFC; Ma et al. 2003). The muscarinic-induced change in GABAergic signal in the auditory cortex seems to be caused by mechanisms different from those observed in the PFC. Although we were unable to detect any change in the mIPSCs mean amplitude or frequency in the PFC and the auditory cortex, as we already reported for the muscarinic modulation of glutamate release (Atzori et al. 2005).

**Muscarnic receptors modulate N- and P/Q-type calcium channels in GABAergic synaptic terminals**

In the mammalian CNS, the N- and P/Q-type channels contribute to synaptic transmission (Arii et al. 1999; Mochida et al. 1998; Sinha et al. 1997; Westenbroek et al. 1998; Wu and Saggau 1997). We examined the role of N- and P/Q-types channels in GABA release in layer II/III of the auditory cortex. Evoked IPSCs were sensitive to both \(\omega\)-Ctx GVIA and \(\omega\)-Aga TK, indicating that both N-type and P/Q-type calcium channels are likely to participate in GABA release from auditory cortex terminals. The contribution of P/Q type calcium channels was nearly 80%, whereas the contribution of N-type calcium channels was 45%, suggesting that P/Q type calcium currents, which are the only Ca\(^{2+}\)-channels inducing neurotransmitter release in fast-spiking interneurons (Zaitsev et al. 2007), predominantly mediate GABA release in these synapses. The finding that the sum of N- and P/Q-type calcium channels blockade corresponded to >100% inhibition of synaptic transmission is in agreement with previous studies (Lei and McBurnay 2003; Mintz et al. 1995; Salgado et al. 2005; Wheeler et al. 1996) and is most likely caused by the supralinear relationship between calcium influx and neurotransmitter release (Dodge and Rahamimoff 1967a,b).

The presence of the specific Ca-channel blockers \(\omega\)-Ctx GVIA or \(\omega\)-Aga TK greatly impaired the effectiveness of oxotremorine in decreasing eIPSCs amplitude. N-type or P/Q-type Ca\(^{2+}\) channel blockers inhibited nearly 50% of the presynaptic inhibition produced by muscarinic receptor activation, suggesting that both N-type and P/Q-type calcium channels are the major (direct or indirect) mediators of the muscarinic eIPSC reduction.

**M_{2}Rs give the largest contribution to the muscarinic GABAergic depression**

Different subtypes of muscarinic receptors (M_{1,4}Rs) mediate muscarinic presynaptic inhibition (Fukudome et al. 2004; Li et al. 2004; Perez-Rosello et al. 2005). The recent discovery of toxins acting selectively on different muscarinic receptors [muscarinic toxins (MTs)] (Jerusalinsky and Harvey 1994; Karlsson et al. 2000) helped to dissect the otherwise potentially inaccurate pharmacology of the muscarinic-induced eIPSC

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**Fig. 8.** P/Q channels are modulated by both M_{1} and M_{2} receptors. Recordings performed in the presence of the N-type Ca\(^{2+}\) channel blocker \(\omega\)-Ctx GVIA (1 \(\mu\)M), leaving intact the P/Q type component of GABA release. A: presence of M_{1}R blocker MT-7 greatly reduces oxotremorine-induced eIPSC depression (time-course, representative traces in inset). B: presence of M_{2}R blocker AF-DX 116 reduces only modestly oxotremorine-induced eIPSC depression (representative trace on right). C: mean \pm SE of eIPSC amplitude reduction. Data suggest that P/Q Ca\(^{2+}\) channel-related component of GABA release is most sensitive to M_{1}R and less sensitive to M_{2}R activation.

Different class than those generating eIPSCs, these data do not favor the possibility of a postsynaptic locus of action for the muscarinic agonists.
FIG. 9. PI3K and Ca2+/H11001-independent PKC are responsible for most of the muscarinic-induced eIPSC decrease. Effect of slice preincubation with different drugs on the oxotremorine-induced eIPSC modulation. Each letter displays the time-course and representative traces of eIPSC from slices preincubated for ≥2 h with the following: (A) PLC blocker U73122 (10 μM), (B) PI3K blockers wortmannin (200 nM), (C) LY294002 (1 μM), (D) nonspecific PKC blocker bisindolylmaleimide (1 μM), (E) Go 6976, specific blocker of the Ca2+/H11001-dependent PKC, and (F) Go 6976 + the M2R blocker AF-DX 116. G: bar graph showing mean ± SE of oxotremorine-induced depression of eIPSC amplitude after slice preincubation with drugs disrupting different intracellular second-messenger pathways. Although 8- to 12-h pertussis toxin (PTX) incubation had no effect, 14- to 18-h incubation did block significantly the muscarinic eIPSC reduction, although to a small degree, possibly because of its slow kinetic action. Similarly, U73122 affected muscarinic eIPSC depression only to a small extent, whereas wortmannin or LY294002 blocked it almost completely. Modest block of the oxotremorine modulation in the presence of U73122 suggests the additional presence of an alternative PLC-independent pathway. The almost complete block of oxotremorine effect by wortmannin or LY294002 indicates a PI3K involvement in the corresponding second-messenger cascade. The nonspecific PKC blocker bisindolylmaleimide completely blocks the oxo-induced eIPSC depression, whereas Go 6976 slightly blocks oxotremorine effect when it acts alone but not in the presence of the M2R blocker AF-DX 116. Data suggest that M1Rs and M2Rs are associated, respectively, with the activation of a Ca2+/H11001-dependent and a Ca2+/H11001-independent PKC.
decrease. Despite the common tenet that most cortical muscarinic receptors belong to the M₁ or M₅ families, the modest or null effect of the agonist and antagonist of M₁Rs and antagonist of M₅Rs raised the doubt that neither receptor type played a major role in the muscarinic-induced eIPSC decrease. This was confirmed when the presence of either of two specific blockers of M₅Rs completely blocked the muscarinic-induced eIPSC decrease. Co-localization of M₅Rs with the GABAergic interneuronal marker PV in the axon terminals and in the somata (Chaudhuri et al. 2005; Hajos et al. 1998). Our study suggested that both M₁R and M₂R modulate GABA release in GABAergic cells, similar to recent findings in the visual cortex of the monkey (Disney et al. 2006). The extent of the eIPSC depression induced by the specific M₁R agonist and blocked by the M₁ antagonist indicates that the remaining contribution to the muscarinicergic eIPSC decrease (about one third of the total eIPSC depression) is associated with the activation of M₅Rs.

Several recent studies have shown the presence of M₅Rs in GABAergic interneurons. In particular, two regions adjacent to the temporal cortex, namely the hippocampus and the entorhinal cortex, display co-localization of M₅Rs with the GABAergic interneuronal marker PV in the axon terminals and in the somata (Chaudhuri et al. 2005; Hajos et al. 1998). Our study revealed that one function of M₅Rs in the auditory cortex is the induction of a dramatic decrease of GABA release without directly affecting the interneuronal presynaptic release machinery. Because the ω-CgTxGVIA–insensitive component of GABA release is presumably represented by P/Q channels, our results suggested that the component of the muscarinic reduction of the GABA release mediated by P/Q of channels is mediated for the most part by M₅Rs located in fast-spiking neurons (Zaitsev et al. 2007) and only modestly by M₅Rs (Fig. 8). A corollary of the preceding results is that, different from P/Q channels, the blockage of the ω-CgTxGVIA–sensitive component of GABA release (N-type channels) is probably mediated completely by activation of M₅Rs.

Metabolic pathway

The great effectiveness of the PKC blocker bisindolylmaleimide in preventing the oxotremorine-induced eIPSC block suggested that both M₁R and M₅R modulate GABA release in the auditory cortex by activation of PKC. The involvement of PKC in N- and P/Q-type Ca²⁺ channel modulation has been reported previously by other groups (Perroy et al. 2000; Stefani et al. 2002; Wang et al. 2003). Various isoforms of PKCs are differentially involved in different metabolic pathways and have different sensitivity to Ca²⁺ (Corbalan-Garcia and Gomez-Fernandez 2006). Taking advantage of the existence of a selective drug affecting Ca²⁺-dependent PKC, we showed that the M₁R-dependent eIPSC depression is mediated by Ca²⁺-independent PKC, whereas the muscarinic PLC-dependent eIPSC depression seems to be mediated by Ca²⁺-dependent PKC.

M₂Rs are preferentially coupled to the Gᵢₒ protein family, whose activation reduces adenylate cyclase activity and/or inhibits voltage-gated Ca²⁺-channels (Allen and Brown 1993; Allen et al. 1993; Liu et al. 2003), suggesting a PTX-sensitive mechanism as a possible signaling pathway. On the contrary, the residual M₁R-mediated effect was expected to be preferentially coupled to the hydrolysis of phosphatidylinositol (Eaton 1993), similar to the metabolic cascade which depresses the release of glutamate in the same area (Atzori et al. 2005). In agreement with the results obtained with the muscarinic toxins, we found that the inhibition of PLC by U73122 occludes an approximately one-third part of modulation by oxotremorine, confirming that activation of typical PLC-associated M₁Rs is in part responsible for the modulation of GABA release. The muscarinic eIPSC inhibition was blocked by PTX, as expected for the M₁R-mediated process coupled to G-proteins of the Gᵢₒ type (Zhang et al. 2002). The modest reduction by PTX in blocking the muscarinic effect was most likely caused by the incomplete effect of the toxin, whose full extent would require an administration time exceeding the viability of a slice preparation. In smooth myocytes, the activation of muscarinic M₁Rs is coupled to the PI₃K–PKC pathway (Callaghan et al. 2004). The great extent of the block of the muscarinic eIPSC depression after preincubation with different selective PI₃K inhibitors suggests that, also in this case, the PI₃K takes part in the M₁R pathway.

Our experiments did not allow us to determine whether the activation of PI₃K by M₂Rs and that of PKC by PI₃K are direct or indirect or whether or not one type only of GABAergic fibers carried M₁Rs and M₅Rs at the same time, although the absence of eIPSC effect by the N-type channel blocker ω-CgTxGVIA in some recordings suggested the possibility that two population of axonal fibers exist: one void of N-type channels and another containing both N- and P/Q-type Ca²⁺-channels, similar to a recent finding in the hippocampus (Poncer et al. 2000). Further studies are needed to address this question conclusively.

Functional significance

Several nonmutually exclusive hypotheses could account for a possible physiological significance of the muscarinic-induced decrease in inhibition. One of them could be the promotion of the transition between the sleep and wake states. In fact, the extent of the cholinergic influence in auditory cortical processing depends on the alertness of the animal: during slow-wave sleep, the virtual absence of acetylcholine from the cortex would minimize single cell excitability by allowing a maximal K⁺ neuronal conductance (Krnjevic 1993) and maximizing the effectiveness of GABAergic inhibitory currents associated with the thalamic spindles (Lee and McCormick 1997; McCormick 1993; McCormick and Prince 1986). Tonic activation of the corticopetal cholinergic NB produced during the sleep-to-wake transition would produce a basal cholinergic tone activating high-affinity M₅Rs on GABAergic interneurons, inhibiting wave-like release of GABA associated with the sleep state, and increasing the overall responsiveness of the auditory cortex to sensory stimuli.

Another possibility is that the decrease of GABA release after the activation of M₅Rs in GABAergic axons is a phasic function associated with novelty and attention (Sarter et al. 2005a,b). Studies in different sensory cortices report a transient increase in the responsiveness to sensory stimuli associated with the activation of muscarinic receptors. The muscarinic-induced decrease of GABA release might potently increase the excitability of a particular area of the auditory cortex by enhancing its sensitivity to corticopetal thalamic input at the
best frequency in conjunction with nicotinic effects (Hsieh et al. 2000), as well as to cortico-cortical stimuli originating in auditory cortical regions with different best frequencies (Kaur et al. 2005; Metherate et al. 2005).

A third hypothesis, nonmutually exclusive with the previous ones, is that the decrease in glutamate and GABA release associated with the presence of acetylcholine would change the balance between auditory input processed by high-probability synapses versus low-probability synapses, thus favoring a more sophisticated processing of envelope-related information (Atzori et al. 2001). This hypothesis would account for a less effective analysis of auditory information in the case of impaired cholinergic function like Alzheimer disease (Mahendra et al. 2005).

The possibility of the existence of two anatomically and metabolically segregated pathways associated with the decrease of GABA release in functionally different classes of cortical GABAAergic interneurons is a hypothesis that needs further exploration. We conclude that the activation of M1Rs after the activation of cholinergic corticopetal fibers supplies a potent and reversible mean to transiently regulate cortical excitability by decreasing GABA release through the activation of the PI3,PKC (Ca2+-independent) metabolic pathway and the activation of M1Rs through the PLC/PKC (Ca2+-dependent) pathway.

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