Methyllycaconitine Is a Potent Antagonist of $\alpha$-Conotoxin-MII-Sensitive Presynaptic Nicotinic Acetylcholine Receptors in Rat Striatum

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ABSTRACT

The plant alkaloid methyllycaconitine (MLA) is considered to be a selective antagonist of the $\alpha$7 subtype of neuronal nicotinic acetylcholine receptor (nAChR). However, 50 nM MLA partially inhibited (by 16%) $[^{3}H]$dopamine release from rat striatal synaptosomes stimulated with 10 $\mu$M nicotine. Other $\alpha$7-selective antagonists had no effect. Similarly, MLA (50 nM) inhibited $[^{3}H]$dopamine release evoked by the partial agonist (2-chloro-5-pyridyl)-9-azabicyclo[4.2.1]non-2-ene (UB-165) (0.2 $\mu$M) by 37%. In both cases, inhibition by MLA was surmountable with higher agonist concentrations, indicative of a competitive interaction. At least two subtypes of presynaptic nAChR can modulate dopamine release in the striatum, and these nAChR are distinguished by their differential sensitivity to $\alpha$-conotoxin-MII ($\alpha$-CTx-MII). MLA was not additive with a maximally effective concentration of $\alpha$-CTx-MII (100 nM) in inhibiting $[^{3}H]$dopamine release elicited by 10 $\mu$M nicotine or 0.2 $\mu$M UB-165, suggesting that both toxins act at the same site. This was confirmed in quantitative binding assays with $^{125}$I-$\alpha$-CTx-MII, which displayed saturable specific binding to rat striatum and nucleus accumbens with $B_{max}$ values of 9.8 and 16.5 fmol/mg of protein, and $K_{d}$ values of 0.63 and 0.83 nM, respectively. MLA fully inhibited $^{125}$I-$\alpha$-CTx-MII binding to striatum and nucleus accumbens with a $K_{d}$ value of 33 nM, consistent with the potency observed in the functional assays. We speculate that MLA and $\alpha$-CTx-MII interact with a presynaptic nAChR of subunit composition $\alpha3/\alpha6/\beta2\beta3$ on dopamine neurons. The use of MLA as an $\alpha$7-selective antagonist should be exercised with caution, especially in studies of nAChR in basal ganglia.

Nicotinic acetylcholine receptors (nAChR) are modulators of neuronal function in the central nervous system (Role and Berg, 1996). One way in which nAChR can modify neuronal activity is by facilitating neurotransmitter release, and this may be accomplished by presynaptic or somatodendritic nAChR (Wonnacott, 1997). Dopamine release in rodent striatum may be modulated by presynaptic nAChR on dopamine terminals (Wonnacott, 1997; Zhou et al., 2001) as well as somatodendritic nAChR on dopamine neurons in the substantia nigra (Clarke et al., 1987; Reuben et al., 2000; Klink et al., 2001). The nigrostriatal system has merited attention because of its relevance to the motor stimulant and addictive properties of nicotine and because these nAChR represent potential therapeutic targets for the treatment of Parkinson’s disease.

Neuronal nAChR are pentameric ligand-gated cation channels, comprised of one or more different subunits, from a portfolio of nine $\alpha$ ($\alpha2$–$\alpha10$) and three $\beta$ ($\beta2$–$\beta4$) subunits expressed in the nervous system (Lukas et al., 1999; Elgoyhen et al., 2001). Determination of the subunit composition of native nAChR presents a major challenge. This endeavor is hampered by a lack of subtype-selective nicotinic ligands, the majority of those available are antagonists derived from natural products. The $\alpha$-conotoxins comprise a family of peptide toxins elaborated by Conus sp. and directed against particular nAChR subtypes (McIntosh et al., 1999). From studies of functional nAChR expressed in Xenopus oocytes, $\alpha$-conotoxin-MII ($\alpha$-CTx-MII) was originally defined as a specific antagonist of nAChR composed of $\alpha3$ and $\beta2$ subunits (Cartier et al., 1996; Harvey et al., 1997). $^{125}$I-$\alpha$-CTx-MII labels a unique population of nicotinic binding sites in mouse...
and monkey brain (Whiteaker et al., 2000; Quik et al., 2001). This toxin partially blocks $^{3}$H]dopamine release from rat striatal synaptosomes stimulated with nicotine (Kulak et al., 1997) or anatoxin-a (Kaiser et al., 1998), providing evidence for the heterogeneity of presynaptic nAChR on dopamine terminals, with one population containing an $\alpha 3\beta 2$ interface. Studies with the novel partial agonist UB-165 led us to propose an $\alpha 4\beta 2^*$ nAChR subtype as a candidate for the other nAChR population (Sharples et al., 2000). The contribution of the $\beta 2$ subunit to both nAChR subunits is consistent with the localization of $\beta 2$ nAChR subunit immunoreactivity in most dopaminergic terminals in the dorsal striatum (Jones et al., 2001) and the absence of nicotine-evoked dopamine release from striatal synaptosomes or slices prepared from $\beta 2$ null mutant mice (Grady et al., 2001; Zhou et al., 2001). However, the subunit composition of striatal presynaptic nAChR is likely to be more complex than pairwise combinations of subunits and this is denoted by the asterisk (Lukas et al., 1999).

Midbrain dopamine neurons express most nAChR subunits (Klink et al., 2001), including a particularly high expression of mRNA corresponding to the $\alpha 6$ and $\beta 3$ nAChR subunits (Le Novère et al., 1996). Patch-clamp recording and single cell polymerase chain reaction analysis of rat midbrain dopamine neurons, and comparison with data from transgenic mice deficient in a particular nAChR subunit, led to the tentative subunit compositions $\alpha 4\alpha 6/\alpha 3\alpha 5(\beta 2)_2$ and $(\alpha 4)2\alpha 5(\beta 2)_2$ for two major nAChR subtypes found on cell bodies of these neurons in the substantia nigra and ventral tegmentum (Klink et al., 2001). Typical fast desensitizing $\alpha 7$-type nAChR currents are also observed in some midbrain dopamine neurons (Pidoplichko et al., 1997; Klink et al., 2001), but $\alpha 7^*$ nAChR do not seem to mediate $^{3}$H]dopamine release from striatal synaptosomes as the $\alpha 7$-selective antagonists $\alpha$-bungarotoxin ($\alpha$Bgt) and $\alpha$-conotoxin-ImI ($\alpha$-CTx-ImI) are without effect (Rapier et al., 1990; Kulak et al., 1997). However, we previously noted that 50 nM methyllycaconitine (MLA), a potent $\alpha 7$-selective antagonist, produced a partial inhibition of anatoxin-a-evoked $^{3}$H]dopamine release from striatal synaptosomes (Kaiser and Wonnacott, 2000), in agreement with the previous observation of Clarke and Reuben (1996). Klink et al. (2001) have also shown that low nanomolar concentrations of MLA inhibit a population of non-$\alpha 7$ nAChR currents, correlated with the tentative subunit composition $\alpha 4\alpha 6\alpha 5(\beta 2)_2$, in the cell bodies of mesencephalic dopamine neurons.

MLA is a hexacyclic norditerpenoid alkaloid, isolated from Delphinium sp. (Wonnacott et al., 1993). MLA is a competitive nicotinic antagonist with approximately 100-, 1,000-, and 10,000-fold higher affinity for $\alpha 7$ nAChR, compared with $\alpha 3\beta 2$, $\alpha 4\beta 2$, and muscle nAChR, respectively (Alkondon et al., 1992; Drasdo et al., 1992; Wonnacott et al., 1993). The pharmacology and distribution of $^{3}$H[MLA binding sites in rodent brain tissue corresponds well with that of $^{125}$I-$\beta$gt binding sites (Davies et al., 1999; Whiteaker et al., 1999), and sensitivity to low nanomolar concentrations of MLA has been interpreted as evidence for $\alpha 7$ nAChR. However, in avian neuronal preparations discrepancies between MLA- and $\alpha$Bgt-sensitive responses have been reported (Yum et al., 1996; Yu and Role, 1998), raising the possibility that minority populations of neuronal nAChR with particular subunit combinations may show differential sensitivity to these two toxins.

In this study, we have characterized pharmacologically the MLA-sensitive portion of nAChR-mediated $^{3}$H]dopamine release from striatal synaptosomes. We show that nanomolar concentrations of MLA inhibit the same nAChR population as $\alpha$-CTx-MII. Using $^{125}$I-$\alpha$-CTx-MII to label nAChR directly, we confirm that MLA potently interacts with this site. The relationship between presynaptic and somatodendritic nAChR on dopamine neurons, with respect to subunit composition, is discussed.

**Materials and Methods**

Sprague-Dawley rats were obtained from the University of Bath Animal House (Bath, UK) breeding colony and the Health Sciences Center (Denver, CO). $^{7,8}$H]Dopamine ($^{3}$H]dopamine, specific activity 1.78 TBq/mmol) and $^{125}$I autoradiographic microscales were purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). $\alpha$-CTx-MII was provided by Tocris Cookson (Bristol, UK). $\alpha$-CTx-ImI, MLA, $^{(\pm)}$nicotine, $\alpha$Bgt, mecamylamine, pargyline, and nomifensine were obtained from Sigma Chemical (Poole, Dorset, UK). $^{(\pm)}$UB-165 was synthesized by Professor T. Gallagher (University of Bristol, Bristol, UK) as described previously (Wright et al., 1997). $^{125}$I-$\alpha$-CTx-MII was synthesized by addition of an N-terminal tyrosine and subsequent iodination, as described by Whiteaker et al. (2000). All other chemicals used were of analytical grade and obtained from standard commercial sources.

**Superfusion of Synaptosomes.** $^{3}$H]Dopamine release from rat striatal synaptosomes was measured as described previously (Kaiser et al., 1998). In brief, male Sprague-Dawley rats (250–350 g) were killed by cervical dislocation and decapitated, and striata (including dorsal striatum and nucleus accumbens; 180–240 mg wet tissue/rat) were rapidly dissected. P2 synaptosomes were obtained by homogenization followed by differential centrifugation and were loaded with $^{3}$H]dopamine (0.1 $\mu$M, 0.132 MBq/ml) for 15 min at 37°C. Synaptosomes were deposited on GF/B filter discs (Whatman, Maidstone, UK) in open chambers of a superfusion apparatus (model SF-12; Brandel, Gaithersburg, MD), with striata from two rats providing enough tissue for 12 superfusion chambers. Synaptosomes were superfused with Krebs-bicarbonate buffer of the following composition: 118 mM NaCl, 2.4 mM KCl, 2.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 25 mM NaHCO$_3$, and 10 mM glucose, pH 7.4, saturated with 95% O$_2$, 5% CO$_2$, and supplemented with 1 mM ascorbic acid, 8 $\mu$M pargyline, and 0.5 $\mu$M nomifensine to prevent dopamine degradation and reuptake, respectively. The flow rate was 0.5 ml/min. After a 20-min washout period, the synaptosomes were superfused for a further 10 min in Krebs’ buffer with or without antagonist (except in the case of $\alpha$Bgt, which was present in the perfusate for 1 h). The nicotinic agonists $^{(\pm)}$nicotine (10 or 100 $\mu$M) or $^{(\pm)}$-UB-165 (0.2 or 1 $\mu$M) were then applied for 40 s, alone or in combination with antagonist, separated from the bulk buffer flow by 10-s air bubbles. Two-minute fractions were collected and counted in a TRI-CARB liquid scintillation analyzer (model 1600, counting efficiency 48%; Packard Instrument Company, Inc., Downers Grove, IL).

Radioactivity remaining in the synaptosomes at the end of the experiment was determined by counting the filters from the superfusion chambers. Total radioactivity present in synaptosomes at the time of agonist stimulation was calculated as the sum of subsequently released $^{3}$H]dopamine plus radioactivity remaining on the filters.

**Superfusion Data Analysis.** Evoked tritium release above baseline was calculated as a percentage of the total radioactivity present in the synaptosomes immediately before stimulation. The baseline was derived, using SigmaPlot version 2.0 (Jandel Scientific, San Rafael, CA), by fitting the following double exponential decay equation to the data: $y = ae^{-bx} + ce^{-dx}$, where $a$ and $c$ are initial (at $x = $ 0) and final counts per minute, respectively, and $b$ and $d$ are the rate constants (seconds$^{-1}$) for the fast and slow decay phases, respectively.
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0) release in each phase, \(b\) and \(d\) are the decay constants in each phase, and \(x\) is the fraction number.

Data are expressed as percentages of the corresponding controls, assayed in parallel in the absence of antagonist, and are the mean ± S.E.M. of several independent experiments, each consisting of two to three replicate chambers for each condition in each experiment. Statistical analyses were performed using the unpaired Student’s \(t\) test, and one-way ANOVA with Tukey’s post hoc test.

**Quantitative Autoradiography of \(125^I\)-a-CTX-MII Binding.**

\(125^I\)-a-CTX-MII binding was assessed by quantitative autoradiography. The methods used were similar to those detailed in Whiteaker et al. (2000). Six male Sprague-Dawley rats (300–350 g) were killed by cervical dislocation, and the brains were removed from the skull and rapidly frozen by immersion in isopentane (−35°C, 30 s). The frozen brains were wrapped in aluminum foil, packed in ice, and stored at −70°C until sectioning. Tissue sections (20 µm in thickness) were prepared, using a Leica CM1850 cryostat/microtome refrigerated to −23°C, and thaw mounted onto subbed microscope slides (Richard Allen, Richland, MI). Slides were subbed by incubation with gelatin (1% w/v)/chromium aluminum sulfate (0.1% w/v) for 2 min at 37°C; drying overnight at 37°C, incubation at 37°C for 30 min in 0.1% (w/v) poly-l-lysine in 25 mM Tris, pH 8.0; and drying at 37°C overnight. Mounted sections were stored, desiccated, at −70°C until use. Twelve series of sections were collected from three brains for use in the saturation binding experiments, with 15 series being collected from the remaining brains for use in the inhibition binding experiments. Sections were collected from the front to the back of the striatum (approximately +2.2 mm to −1.8 mm relative to bregma).

Before incubation with \(125^I\)-a-CTX-MII, sections were incubated in binding buffer [144 mM NaCl, 1.5 mM KCl, 2 mM CaCl\(_2\), 1 mM MgSO\(_4\), 20 mM HEPEPS, and 0.1% (w/v) bovine serum albumin, pH 7.5] containing PMSF (1 mM; to inactivate endogenous serine proteases) at 22°C for 15 min. For all \(125^I\)-a-CTX-MII binding reactions, the standard binding buffer was also supplemented with 5 mM EDTA, 5 mM EGTA, and 10 µg/ml each of aprotinin, leupeptin, trypsin, soybean trypsin inhibitor, and pepstatin A to protect the ligand from endogenous proteases.

Saturation binding experiments were performed by incubation of six series of sections with concentrations of \(125^I\)-a-CTX-MII varying between 0.05 and 2.5 nM to determine total binding at each concentration for 2 h at 22°C. Nonspecific binding (in the presence of 10 nM (−)nicotine) was determined in a series of adjacent sections, and receptor-specific binding was determined as the difference between total and nonspecific binding at each concentration. The ability of MLA to inhibit \(125^I\)-a-CTX-MII binding was determined by coincubation of sections with 0.5 nM \(125^I\)-a-CTX-MII and varying concentrations of MLA (1–1000 nM) for 2 h at 22°C. After incubation with \(125^I\)-a-CTX-MII, the slides were washed as follows: 60 s in binding buffer (twice at 22°C), 10 s in 0.1X binding buffer (twice at 0°C), and twice at 0°C for 10 s in 5 mM HEPEPS, pH 7.5. Sections were initially dried with a stream of air then by overnight storage at 22°C under vacuum.

Mounted, desiccated sections were apposed to film (3 days, Hyperfilm β-Max film; Amershams Biosciences UK). To allow quantitation, each film was also exposed to radioactivity microscopes. After the films had been exposed they were developed and signal intensity in selected brain regions was measured by digital image analysis. Films were illuminated using a Northern Light light box, and autoradiographic images of the sections and standards were captured using a charge-coupled device imager camera. Signal intensity was determined using NIH Image 1.61. Six independent measurements from different tissue sections were made for nucleus accumbens and striatum, under each incubation condition, for each rat. The optical density measurements for each brain area were averaged, and the mean optical density was used to calculate the degree of labeling by reference to the relevant standard curve.

**Results**

MLA Partially Inhibits Nicotine-Evoked \([^3H]\)Dopamine Release from Striatal Synaptosomes. Nicotine-evoked \([^3H]\)dopamine release from rat striatal synaptosomes was monitored in the presence and absence of a variety of nicotinic antagonists. Nicotine (10 µM; delivered as a 40-s pulse) elicited a peak of radioactivity above basal release, and this was almost totally abolished in the presence of the general nAChR antagonist mecamylamine (10 µM; Fig. 1A). The residual release was equivalent to the nonspecific release observed when agonist was replaced with buffer. In the presence of MLA (50 nM), a small decrease in the amount of nicotine-evoked \([^3H]\)dopamine release was consistently observed (Fig. 1, A and B).

The concentration-response relationship for the effect of MLA on \([^3H]\)dopamine release evoked by 10 µM nicotine (Fig. 1B) showed a significant \((p < 0.01)\) inhibition of the response between 30 and 100 nM MLA. Higher concentrations of MLA were not examined because MLA becomes nonselective above 100 nM and would interact with various non-\(\alpha7\) subtypes of nAChR (Drasdo et al., 1992; Wonnacott et al., 1993). At 10 nM MLA, a concentration frequently used to inhibit \(\alpha7\) nAChR, there was a small but nonsignificant reduction in the response to 10 µM nicotine.

MLA (50 nM) was compared with other \(\alpha7\)-selective nAChR antagonists for inhibition of nicotine-evoked \([^3H]\)dopamine release from rat striatal synaptosomes (Fig. 1C). Nicotine at a maximally effective (100 µM) and a submaximal concentration (10 µM; Fig. 1C, inset) were compared. At the higher concentration, nicotine elicited approximately 50% more \([^3H]\)dopamine release than 10 µM nicotine; in both cases the responses were decreased to the level of a buffer control in the presence of mecamylamine (10 µM). MLA significantly inhibited \([^3H]\)dopamine release evoked by 10 µM nicotine, by 16.3 ± 5.5% \((n = 4, p < 0.05)\) but had no significant effect on release evoked by the higher concentration of nicotine (Fig. 1C). However, maximally effective concentrations of the \(\alpha7\)-selective nAChR antagonists αBgt (40 nM) and α-CTx-ImI (1 µM; Pereira et al., 1996) did not diminish \([^3H]\)dopamine release from striatal synaptosomes stimulated with either concentration of nicotine.

The inhibition by MLA, but not by the other \(\alpha7\) nAChR antagonists, suggested that MLA was interacting with a non-\(\alpha7\) nAChR. To explore this possibility, other nAChR antagonists were used to clarify the subtype of nAChR involved. In the presence of 100 nM α-CTx-MII, a concentration that has been shown to selectively and maximally inhibit \(\alpha3\beta2\) nAChR (Cartier et al., 1996; Kaiser et al., 1998), \([^3H]\)dopamine release evoked by 10 µM nicotine was decreased by 35.5 ± 4.9% \((n = 3, p < 0.01;\text{ Fig. 2})\). MLA also
partially inhibited nicotine-evoked \(^{3}H\)dopamine release, consistent with previous experiments (discussed above), but when coapplied with \(\alpha\)-CTx-MII no additional inhibition of \(^{3}H\)dopamine release was observed (26.2 \pm 3.2\% inhibition, \(n = 3, p < 0.01\); Fig. 2).

The antagonist DH\(\beta\)E has a broad specificity for neuronal nAChR, but is more selective for non-\(\alpha\)-7-containing nAChR at low concentrations (1 \(\mu\)M; Harvey et al., 1996). At 1 \(\mu\)M, DH\(\beta\)E produced a substantial inhibition (62.7 \pm 6.7\%) of \(^{3}H\)dopamine release evoked by 10 \(\mu\)M nicotine. Neither \(\alpha\)-CTx-MII (100 nM) nor MLA (50 nM) in combination with DH\(\beta\)E (1 \(\mu\)M) showed any additivity with respect to the extent of inhibition observed (Fig. 2).

Subtype-selective agonists that activate only one of the nAChR subtypes present on striatal dopamine terminals would provide another approach to characterizing the action of MLA on striatal synaptosomes. We recently characterized the novel synthetic agonist UB-165 (Sharples et al., 2000),

Fig. 1. Inhibition by MLA of nicotine-evoked \(^{3}H\)dopamine release from striatal synaptosomes. Synaptosomes were loaded with \(^{3}H\)dopamine and superfused as described under Materials and Methods; 2-min (1-ml) fractions were collected and counted for radioactivity. Antagonist was introduced into the perfusing buffer 10 min before stimulation for 40 s with nicotine, except in the case of Bgt in which synaptosomes were incubated with it for 1 h before addition of agonist. A, release of \(^{3}H\)dopamine over time, showing the response to 10 \(\mu\)M nicotine (arrow) in the absence (○) and presence (□) of 50 nM MLA, or 10 \(\mu\)M mecamylamine (●). Evoked \(^{3}H\)dopamine release is expressed as a percentage of the mean basal release; values are the mean \pm S.E.M. of the triplicate chambers from a representative experiment. B, effect of increasing concentrations of MLA on nicotine-evoked dopamine release. Synaptosomes were stimulated with 10 \(\mu\)M nicotine, in the presence and absence of MLA. Data are expressed as a percentage of the response to 10 \(\mu\)M nicotine alone. Error bars indicate the S.E.M. from at least three separate experiments, each consisting of two or more replicate chambers (\(**, p < 0.01\), significantly different from control response; \(††, p < 0.05\), significantly different from response obtained in the presence of 30 and 50 nM MLA; one-way ANOVA, Tukey’s post hoc test). C, effect of the \(\alpha\)-7-selective antagonists Bgt (40 nM), MLA (50 nM), \(\alpha\)-CTx ImI (1 \(\mu\)M), and the general nAChR antagonist mecamylamine (10 \(\mu\)M) was compared for their effect on \(^{3}H\)dopamine release evoked by 10 \(\mu\)M nicotine (○) or 100 \(\mu\)M nicotine (●). Values are expressed as a percentage of the response to 10 \(\mu\)M nicotine, in the absence of antagonist, determined in parallel. Inset, concentration-response relationship for nicotine-evoked \(^{3}H\)dopamine release from striatal synaptosomes, expressed as a percentage of the response to 10 \(\mu\)M nicotine. Values are the mean \pm S.E.M. from at least three separate experiments, each consisting of two or more replicate chambers (\(**, p < 0.01\), significantly different from control response; one-way ANOVA, Tukey’s post hoc test)
alone and in combination with MLA and non-α7 antagonists. Synaptosomes were loaded with [3H]dopamine and superfused as described under Materials and Methods. Antagonists (50 nM MLA, 100 nM α-CTx-MII, and 1 μM DHβE) were introduced into the perfusing buffer, either singly or in combination, 10 min before stimulation for 40 s with 10 μM nicotine. Evoked [3H]dopamine release in the presence of antagonist is expressed as a percentage of the response to 10 μM nicotine alone. Values are the mean ± S.E.M. from at least three independent experiments, each consisting of two or more replicate chambers (⁎⁎, p < 0.01, significantly different from control response; one-way ANOVA, Tukey’s post hoc test; ††, p < 0.01, significantly different from responses obtained with nicotine alone and in combination with α-CTx-MII, MLA, and α-CTx-MII + MLA; one-way ANOVA, Tukey’s post hoc test.) and showed it to be a partial agonist in releasing [3H]dopamine from striatal synaptosomes because it predominately activated the α-CTx-MII-sensitive nAChR subtype. From the effects of MLA on nicotine-evoked [3H]dopamine release (Figs. 1 and 2), we predicted that UB-165-evoked [3H]dopamine release should also be sensitive to low nanomolar concentrations of MLA. We compared two concentrations of UB-165: a maximally effective concentration (1 μM) and one that approximates to its EC50 value (0.2 μM; Sharples et al., 2000).

[3H]Dopamine release evoked by 0.2 and 1 μM UB-165 was 20 and 29%, respectively, of that elicited by 10 μM nicotine (Fig. 3). Mecamylamine (10 μM) reduced the responses of both concentrations of UB-165 to a similar level, approximately 7% of the nicotine response. α-CTx-MII (100 nM) and MLA (50 nM) inhibited [3H]dopamine release evoked by 0.2 μM UB-165 to a similar extent (38.9 ± 3.1 and 37.1 ± 3.9% inhibition, respectively), compared with 53.9 ± 6.0 and 61.3 ± 4.4% inhibition by DHβE (1 μM) and mecamylamine (10 μM), respectively (Fig. 3). Coapplication of α-CTx-MII and MLA produced no additive effect. At the higher agonist concentration (1 μM UB-165), α-CTx-MII and DHβE continued to inhibit the response (by 40.0 ± 7.2 and 57.0 ± 7.1%, respectively) but MLA no longer exerted any significant effect.

Inhibition by MLA of 125I-α-CTx-MII Binding. The effects of MLA on nicotinic agonist-evoked [3H]dopamine release suggest that it acts competitively at the same nAChR subtype as α-CTx-MII. To address this proposition, 125I-α-CTx-MII was used to label nicotinic sites in rat striatum and nucleus accumbens, and the ability of MLA to displace this specific binding was investigated.125I-α-CTx-MII displayed saturable specific binding to striatum and nucleus accumbens (Fig. 4A), with Bmax values of 9.8 ± 1.3 and 16.5 ± 4.6 fmol/mg of protein and KD values of 0.63 ± 0.19 and 0.83 ± 0.55 nM, respectively. Competition assays with serial dilutions of MLA over the range 1 nM to 1 μM were carried out using 0.5 nM 125I-α-CTx-MII, a concentration approximating its KD. MLA was able to fully displace specific binding of the radioligand to both striatum and nucleus accumbens (Fig. 4B), with KD values of 32.9 ± 12.9 and 34.6 ± 13.8 nM, respectively.

Discussion

The present study demonstrates that the presumed α7-selective compound MLA may antagonize other nAChR subtypes found at rat striatal dopaminergic nerve terminals, at concentrations frequently used to selectively block α7

Fig. 2. Inhibition of nicotine-evoked [3H]dopamine release from striatal synaptosomes by MLA and non-α7 antagonists. Synaptosomes were loaded with [3H]dopamine and superfused as described under Materials and Methods. Antagonists (50 nM MLA, 100 nM α-CTx-MII, and 1 μM DHβE) were introduced into the perfusing buffer, either singly or in combination, 10 min before stimulation for 40 s with 10 μM nicotine. Evoked [3H]dopamine release in the presence of antagonist is expressed as a percentage of the response to 10 μM nicotine alone. Values are the mean ± S.E.M. from at least three independent experiments, each consisting of two or more replicate chambers (⁎⁎, p < 0.01, significantly different from control response; one-way ANOVA, Tukey’s post hoc test; ††, p < 0.01, significantly different from responses obtained with nicotine alone and in combination with α-CTx-MII, MLA, and α-CTx-MII + MLA; one-way ANOVA, Tukey’s post hoc test.)

Fig. 3. Sensitivity to MLA of [3H]dopamine release evoked by UB-165. Synaptosomes were loaded with [3H]dopamine and superfused as described under Materials and Methods. Antagonist (50 nM MLA, 100 nM α-CTx-MII, and 10 μM mecamylamine) was added to the perfusing buffer 10 min before stimulation for 40 s with nicotine (10 μM) or UB-165 (0.2 or 1 μM). Results are expressed as a percentage of the response to 1 μM UB-165. Values are the mean ± S.E.M. from at least three independent experiments, each consisting of two or more replicate chambers (⁎⁎, p < 0.01, significantly different from responses obtained with 1 μM UB-165; one-way ANOVA, Tukey’s post hoc test; ††, p < 0.01, †, p < 0.05, significantly different from responses obtained with 0.2 μM UB-165; one-way ANOVA, Tukey’s post hoc test).
nAChR. The ability of 50 nM MLA to partially inhibit nicotinic agonist-evoked [3H]dopamine release was consistently observed at low agonist concentrations, but was surmountable at higher agonist concentrations, suggesting a competitive mode of action. Because none of the other α7-selective compounds examined (αBgt and α-CTx-MII) had any effect on nicotine-evoked [3H]dopamine release from striatal synaptosomes, this strongly argues that MLA is not acting through an α7 nAChR. Moreover, the lack of additivity with α-CTx-MII suggests that both antagonists act at the α-CTx-MII-sensitive nAChR subtype. Confirmation of this interaction was provided by the ability of low nanomolar concentrations of MLA to displace 125I-α-CTx-MII binding to rat striatum and nucleus accumbens.

The absence of a discernible α7 nAChR-mediated component in nicotine-evoked [3H]dopamine release from striatal synaptosomes is well documented (Rapier et al., 1990; Kulak et al., 1997) and substantiated by the lack of effect of αBgt and α-CTx-II in the present study (Fig. 1C). In contrast, we have shown previously that these agents, as well as MLA, antagonize nicotine-evoked [3H]dopamine release from striatal slices, interpreted as evidence for the involvement of an indirect α7 nAChR-mediated component in the slice (Kaiser and Wonnacott, 2000). In the present study, [3H]dopamine release from striatal synaptosomes was examined to focus on presynaptic nAChR localized on the dopamine terminals.

The inhibition by 50 nM MLA of [3H]dopamine release evoked by either nicotine or UB-165 was surmountable (Figs. 1C and 3), consistent with a competitive interaction. Similarly, Clarke and Reuben (1996) observed a surmountable inhibition by MLA of nicotine-evoked [3H]dopamine release from striatal synaptosomes. These authors demonstrated complete inhibition by MLA with an IC50 value of 38 nM, whereas nicotine-evoked [3H]noradrenaline release was notably less sensitive to MLA (IC50 = 1 μM). Full dose-response curves were not determined in the present study because the current appreciation of the heterogeneity of nAChR subtypes governing nicotine-evoked [3H]dopamine release compromises the analysis of such profiles. MLA (50 nM) also partially inhibited (by 37%) [3H]dopamine release from striatal synaptosomes stimulated with 1 μM anatoxin-a, but had no effect when the agonist concentration was increased to 25 μM (Kaiser and Wonnacott, 2000). Thus, MLA potently and competitively inhibits a portion of striatal [3H]dopamine release elicited by a number of nicotinic agonists acting at presynaptic nAChR.

MLA seems to interact with α-CTx-MII-sensitive nAChR because inhibition by the two toxins of nicotine- or UB-165-evoked [3H]dopamine release is not additive when they are applied together (Figs. 2 and 3). This inference is supported by the ability of MLA to potentiate 125I-α-CTx-MII binding. 125I-α-CTx-MII labeled a small population of specific sites to dopaminergic terminals in these brain regions (Quik et al., 2001), assuming 1 mg of protein corresponds to 10 mg of tissue. In the monkey caudate putamen, more than 95% of specific 125I-α-CTx-MII binding sites were lost in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-lesioned animals. Together with the correlation with dopamine transporters, this strongly supports the localization of 125I-α-CTx-MII binding sites to dopaminergic terminals in these brain regions (Quik et al., 2001). αBgt (1 μM) failed to inhibit 125I-α-CTx-MII binding.
binding to mouse striatum, and conversely α-CTx-MII, at concentrations up to 1 μM, did not compete for [125I]-α-Bgt binding to mouse brain membranes (Whiteaker et al., 2000). Thus, the ability of MLA to fully displace [125I]-α-CTx-MII binding (Fig. 4) with a Kᵦ of ~33 nM reflects a non-α7 nAChR activity for this ligand.

The observations in the present study parallel the findings of Klink et al. (2001) for nAChR responses recorded electrophysiologically from dopamine cell bodies in the rat midbrain. These authors showed that in most (75%) dopamine neurons, acetylcholine or nicotine evoked slow whole cell currents that were partially blocked by low nanomolar concentrations of MLA and α-CTx-MII in a nonadditive manner.

This raises the question of the subunit composition of nAChR subtype(s) with which MLA potently interacts. The majority of rat midbrain dopamine neurons express the α3, α4, α5, α6, β2, and β3 subunits (Klink et al., 2001). α-CTx-MII was originally defined as a selective antagonist of nAChR composed of α3 and β2 subunits, with >100-fold lower potency at other pairwise nAChR subunit combinations or α7 nAChR expressed in Xenopus oocytes (Cartier et al., 1996; Harvey et al., 1997; Kaiser et al., 1998). Because binding of CTx-MII to one α3β2 interface would be sufficient for functional inhibition, the toxix specificity was extended to include α3β2-containing (or α3β2⁺) nAChR (Kulak et al., 1997; Kaiser et al., 1998). However, the α3 subunit shares high sequence identity with the α6 subunit (Le Novère and Changeux, 1995) that is highly expressed in dopamine neurons (Le Novère et al., 1996; Goldner et al., 1997; Klink et al., 2001). Deletion of α6 subunit expression by in vivo administration of antisense oligonucleotides decreased nicotine-induced effects on locomotor activity (Le Novère et al., 1999), consistent with a role in motor functions executed by the striatum. The α6 subunit is reluctant to form functional nAChRs in pairwise combination with a β subunit, supporting its participation in more complex subunit assemblies, and the efficient expression of α6 in combination with a variety of mammalian subunits has been demonstrated in heterologous systems (Kuryatov et al., 2000). Immunoisolated α6-containing nAChR from chick retina display moderately high affinity for α-CTx-MII (Kᵦ = 66 nM), but relatively low affinity for MLA (1.3 μM; Vailati et al., 1999).

Expression of the β3 subunit is also limited to a few brain regions, notably catecholaminergic areas where it colocalizes with α6 nAChR subunit expression in the rat brain (Le Novère et al., 1996). Functional nAChRs comprised of rat α3, β2, and β3 subunits and expressed in Xenopus oocytes retain high sensitivity to α-CTx-MII (Luo et al., 2000), whereas transgenic mice lacking expression of the β3 nAChR subunit are deficient in specific binding sites for [125I]-α-CTx-MII in the striatum (Booker et al., 1999). However, chicken immunoisolated β3-containing nAChR that are devoid of α6 subunits do not exhibit high affinity for α-CTx-MII or MLA (Vailati et al., 2000). Thus, the β3 subunit may not confer sensitivity to α-CTx-MII or MLA to nAChR but is crucial for the formation, targeting or stability of α-CTx-MII-sensitive nAChR in basal ganglia in vivo. Klink et al. (2001) propose that the β3 subunit is targeted to terminal regions of dopaminergic neurons and does not participate in somatodendritic nAChR; thus, nAChR at these two locations could exhibit pharmacological (Reuben et al., 2000) and biophysical differences. Indeed, Klink et al. (2001) reported that 1 nM MLA was maximally effective in blocking cell body responses, which is more potent than predicted from the neurochemical measurements of [3H]dopamine release summarized above, or its Kᵦ for interaction with [125I]-α-CTx-MII binding sites (Fig. 4). Although this discrepancy may reflect methodological differences, it could also arise from distinct nAChR subtypes at cell body and terminal locations.

These considerations lead to the proposition that a-CTx-MII- and MLA-sensitive presynaptic nAChRs mediating striatal [3H]dopamine release are comprised of α3 and/or α6 subunits together with β2 and β3 subunits. The finding that [125I]-α-CTx-MII binding to basal ganglia is absent in α6 null mutant mice (Champtiaux et al., 2002), but persists in α3 null mutant mice (Whiteaker et al., 2002), argues for the involvement of the α6 subunit, rather than the α3 subunit, in this nAChR subtype. Klink et al. (2001) proposed the subunit composition α4α6β3β2 for α-CTx-MII- and MLA-sensitive somatodendritic nAChRs. Inclusion of the α4 subunit was based on the absence of responses in midbrain slices from α4 null mutant mice. The ability of nicotinic agonists to elicit dopamine release from striatal synaptosomes prepared from these transgenic animals has not been reported, so no evidence is available for the participation of the α4 subunit in the α-CTx-MII- and MLA-sensitive presynaptic nAChR.

One implication of this and related studies is that sensitivity to mammalian concentrations of MLA should not be considered diagnostic of α7 nAChR, at least in studies of rodent basal ganglia. This is particularly pertinent for in vivo studies, where drug concentrations reaching the nAChR are not known. However, the caveat that must be placed on the nAChR subtype selectivity of MLA may also be exploited for the differentiation of minority subtypes of nAChR, by comparison of sensitivities to MLA and α-CTx-MII.

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