0026-895X/92/040802-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:802-808

Blockade of Nicotinic Currents in Hippocampal Neurons Defines Methyllycaconitine as a Potent and Specific Receptor Antagonist

MANICKAVASAGOM ALKONDON, EDNA F. R. PEREIRA, SUSAN WONNACOTT, and EDSON X. ALBUQUERQUE

Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201 (M.A., E.F.R.P., E.X.A.), Laboratory of Molecular Pharmacology II, Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, RJ 21944, Brazil (E.F.R.P., E.X.A.), and Department of Biochemistry, University of Bath, Bath, BA2 7AY, UK (S.W.)

Received October 23, 1991; Accepted January 24, 1992

SUMMARY

Methyllycaconitine, a toxin isolated from the seeds of *Delphinium brownii*, inhibited acetylcholine- and anatoxin-induced whole-cell currents in cultured fetal rat hippocampal neurons, at picomolar concentrations. This antagonism was specific, concentration dependent, reversible, and voltage independent. Furthermore, methyllycaconitine inhibited ^{125}I-α-bungarotoxin binding to adult rat hippocampal membranes, protected against the α-bungarotoxin-induced pseudoirreversible blockade of nicotinic currents, and shifted the concentration-response curve of acetylcholine to the right in fetal rat hippocampal neurons, suggesting a possible competitive mode of action for this toxin. Remarkably low concentrations of methyllycaconitine (1–1000 fM) decreased the frequency of anatoxin-induced single-channel openings, with no detectable decrease in the mean channel open time. These actions of methyllycaconitine commend this neurotoxin for the characterization of the α-bungarotoxin-sensitive subclass of neuronal nicotinic receptors, which has hitherto eluded functional demonstration.

The characterization of the peripheral nAChR has been greatly enhanced by the availability of potent and selective probes such as α-BGT (1) and histrionicotoxin (2). In the CNS, the study of the nAChR is complicated by the existence of multiple subtypes (3), for which specific pharmacological probes have not yet been identified. Most of the binding studies have relied upon ^{125}I-α-BGT and high affinity nAChR ligands such as [^{3}H]nicotine, [^{3}H]ACh, and [^{3}H]cytisine (4–7), which helped to discriminate two subtypes of nAChR in the brain.

The physiological significance of the binding sites labeled by α-BGT in the mammalian brain (4) remained obscure, because a functional nAChR, sensitive to this toxin, could not be demonstrated for several years. Indeed, functional nAChR identified on the retinal ganglion cells (8), hypophyseal neurons (9), and habenula neurons (10) were found to be insensitive to the blocking action of α-BGT. Recent studies, however, have provided important evidence that the nicotinic responses were blocked by α-BGT in rat hippocampal (11–13) and cerebellar neurons (14), thus supporting the idea that the α-BGT-binding protein is a functional nAChR. The hypothesis that identification of specific antagonists will advance the understanding of the nAChR diversity in the mammalian CNS has been explored in the present study.

MLA (see Fig. 1, *inset*, for structure), a toxin isolated from the seeds of *Delphinium brownii*, has nAChR antagonist properties (15, 16). This novel alkaloid inhibits potently ^{125}I-α-BGT binding to brain membranes, in contrast to its weaker interaction with neuronal nAChR, identified by [^{3}H]nicotine binding, and with muscle nAChR (6, 17). Because of the high affinity and selectivity of MLA for the neuronal α-BGT-sensitive nAChR, we have investigated the actions of MLA on the nAChR of rat hippocampal neurons, using whole-cell and single-channel recordings as well as binding assays. Our results clearly demonstrate that MLA is a potent and specific antagonist of the α-BGT-sensitive nAChR of cultured hippocampal neurons.

Materials and Methods

Hippocampi of fetuses obtained from 17–18-day pregnant rats (Sprague-Dawley) were dissociated according to the procedure described previously (13). The cells were plated at a density of 50,000–70,000 cells/cm^2 on 35-mm culture dishes, which were precoated with collagen. Cultures were used at 12–50 days after plating. Recordings of whole-cell currents were made according to the standard patch-clamp technique (18), using an LM-EPC-7 patch-clamp system. The external solution had the following composition (in mM): NaCl, 165; KCl, 5;

This work was partially supported by grants from the National Institutes of Health (NS-25296) and the United States Army Medical Research and Development Command (DAMD-17-88-C-8119) (E.X.A.), Medical Research Council (G8722675N) (S.W.), and NATO (S.W., E.X.A.).

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor(s); AnTX, (+)-anatoxin-a; ACh, acetylcholine; α-BGT, α-bungarotoxin; MLA, methyllycaconitine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; *d*-TC, *d*-tubocurarine; CNS, central nervous system; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate.

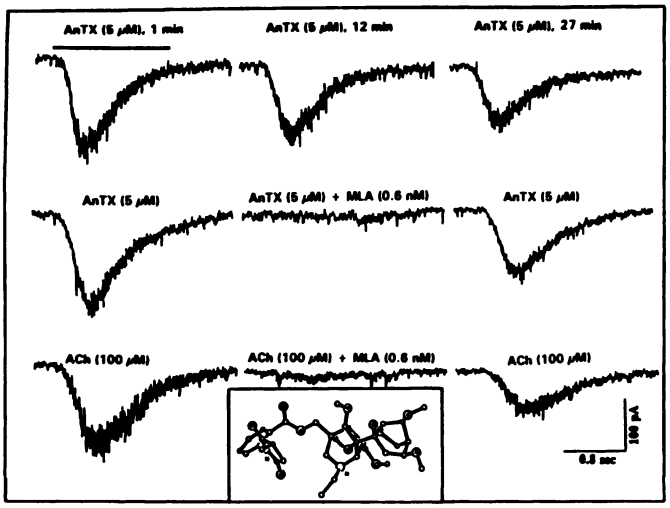

Fig. 1. Samples of whole-cell current recordings evoked by the nicotinic agonists AnTX and ACh in the absence and in the presence of MLA. Fetal hippocampal neurons grown in culture for 32–34 days were used. Holding potential, −50 mV. *Traces in the top row*, a typical control experiment. *Solid line in the first trace*, duration of the pulse used to apply the agonist. A gradual rundown in the amplitude of the responses induced by AnTX was seen in the absence of any test drug over a period of 30 min. A similar rundown was also observed with ACh. *Middle* and *bottom rows*, experiments in which the neuron was exposed to a similar pulse of AnTX (5 μM) or ACh (100 μM) 1 min after the patch was obtained, 12 min after perfusion with 0.6 nM MLA, and 15 min after wash of the cell with normal external solution. The peak amplitude recorded 15 min after MLA was washed out (*middle* and *bottom rows*) is similar to that observed under control conditions 27 min after the patch was obtained. *Inset*, structure of MLA.

$CaCl_2$, 2; HEPES, 5; D-glucose, 10 (pH 7.3, 340 mOsm); plus tetrodotoxin (0.3 μM) and atropine (1 μM). The internal solution consisted of (in mM): CsCl, 80; CsF, 80; Cs-EGTA, 10; and HEPES, 10 (pH 7.3, 330 mOsm). The patch microelectrodes were pulled from borosilicate capillary glass and, when filled with the internal solution, had resistances between 1.5 and 4 MΩ. The seal resistances ranged from 10 to 20 GΩ. The series resistance of the patches was between 5 and 15 MΩ and was not compensated; the voltage drop caused by the series resistance never exceeded 5 mV in these experiments. Whole-cell currents were analyzed using the pCLAMP program (Axon Instruments, Foster City, CA). A U tube positioned close to the neuron (≈50 μm) allowed fast delivery of agonists to both the cell soma and dendrites (19). The solution exchange time constant, as measured using sodium ion concentration jumps in the presence of kainic acid (20), was <25 msec. Increasing the inflow and decreasing the outflow of the solution from the U tube were found to reduce the time constant. However, to avoid a possible desensitization caused by a leak of the agonist from the U tube, the outflow was always kept at least 3 times larger than the inflow. Also, the agonist pulses were applied at intervals of 2 min or more, which permitted a complete recovery of the nAChR from the desensitization induced by a previous pulse of the agonist. The bath solution was exchanged continuously at 2 ml/min, by means of gravity. MLA citrate was a gift from Dr. M. H. Benn (Department of Chemistry, University of Calgary, Alberta, Canada). MLA was dissolved in deionized water to make a stock solution of 1 mM and was kept frozen in small aliquots. On the day of experiment, required final dilutions were made in external solution. MLA was applied to the neurons through the bathing external solution unless otherwise stated. All experiments were performed at room temperature (20–22°).

Single-channel currents were recorded from outside-out patches excised from the cultured fetal rat hippocampal neurons. Both external and internal solutions had the same composition as those used in whole-cell experiments, except that 50 μM DL-aminophosphonovaleric acid was also added to the external medium. After the outside-out patch configuration was established, the recording micropipette was positioned inside a glass minipipe, through which all drugs were delivered. The data were stored on a videocassette tape, filtered at 2 kHz (Bessel), digitized at 12.5 kHz, and analyzed on an IBM computer, using the IPROC-2 program (Axon Instruments). The frequency of channel openings was determined by dividing the total number of openings by the recorded time. A multiple opening was considered as two opening events for the calculation of the frequency.

Washed P2 membranes were prepared, as described previously (6), from hippocampi dissected from the brains of adult male Sprague-Dawley rats, to evaluate the binding of MLA to the nAChR. Aliquots (0.5 ml, approximately 0.5 mg of protein, in 50 mM phosphate buffer, pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride, 0.01% sodium azide, and 1 mM EGTA) were incubated with 10 μl of MLA or competing ligand for 15 min before addition of ^{125}I-α-BGT. Incubation was continued for 2.5 hr at 30°; bound and free radioligand were separated by centrifugation (6). Nonspecific binding, determined in the presence of 1 μM unlabeled α-BGT, was subtracted from the total binding measured.

Results and Discussion

Previous work from this laboratory (11–13) indicated that AnTX and ACh are able to activate nAChR of fetal rat hippocampal neurons. In the present study, we tested the effect of MLA on ACh- and AnTX-activated whole-cell currents in fetal hippocampal neurons at agonist concentrations that were found earlier to evoke 40–60% of the maximal responses. At 600 pM, MLA markedly depressed the whole-cell currents evoked by either AnTX (5 μM) or ACh (100 μM) (Fig. 1). The blockade could be detected at 2 min, and it reached a maximum within 10 min of perfusion of the neurons with MLA-containing external solution. The slowness of blockade (~10 min) obtained with picomolar concentrations (100–600 pM) of MLA could be attributed to the time taken by MLA to reach equilibrium concentrations when applied via the external perfusion medium. When nanomolar concentrations (1–10 nM) of MLA were given in the perfusion solution, it took less time (5–6 min) to achieve a complete blockade of ACh- and AnTX-induced currents. Similarly, when 1 μM MLA was applied together with ACh (100 μM) in short pulses of 1–2-sec duration, through the U tube, almost complete blockade could be seen immediately, even at the first pulse, thus indicating that the onset of MLA action is rapid and diffusion limited. Reversal of MLA blockade (induced by either picomolar bath concentrations or a short pulse of micromolar concentrations) was seen 8–15 min after the neurons were washed with normal external solution. However, it was frequently observed that the peak amplitude of whole-cell currents during the wash phase was smaller than that in the control condition (Fig. 1), even when the washing was continued further. Although the peak current was not restored to pre-MLA levels, it reached levels comparable to control recordings at the equivalent time after a patch was obtained (Fig. 1). Therefore, the blocking action of MLA could be considered completely reversible under these experimental conditions.

MLA blockade was found to be specific to the nAChR of hippocampal neurons. At concentrations that completely blocked AnTX-evoked currents, MLA produced no modification of responses to NMDA, quisqualate, kainate, or GABA (Fig. 2). In fact, even when MLA was tested at concentrations up to 100 nM, the responses of the neurons to NMDA (Fig. 3, *bottom*) and to other glutamate and GABA receptor agonists were unaltered.

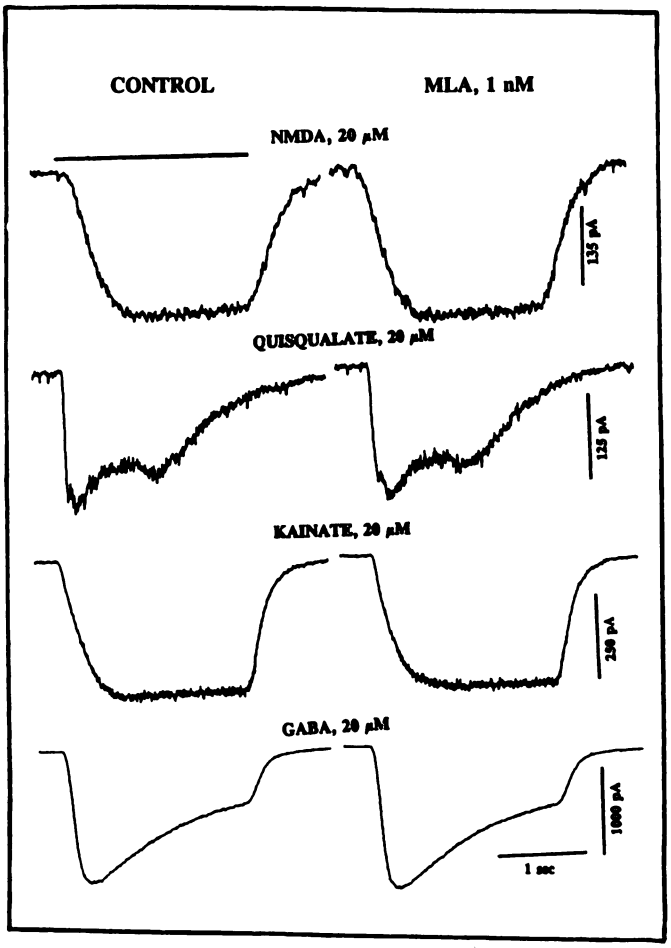

Fig. 2. Samples of whole-cell current recordings evoked by non-nicotinic agonists in the absence and in the presence of 1 nM MLA. All traces were obtained from a single fetal hippocampal neuron grown in culture for 28 days. Holding potential, −50 mV. *Left traces*, control conditions obtained at 5, 7, 9, and 11 min (*top* to *bottom*, respectively) after the patch was established. *Right traces*, recordings made 12, 14, 16, and 18 min (*top* to *bottom*, respectively) after perfusion with 1 nM MLA. Glycine (1 μM) was added together with NMDA, whereas 20 μM DL-aminophosphonovaleric acid was added to both quisqualate and kainate solutions. AnTX (5 μM)-evoked currents in this neuron recorded 3 and 13 min after the patch was established were completely blocked at 8–10 min after perfusion with 1 nM MLA (traces not shown here).

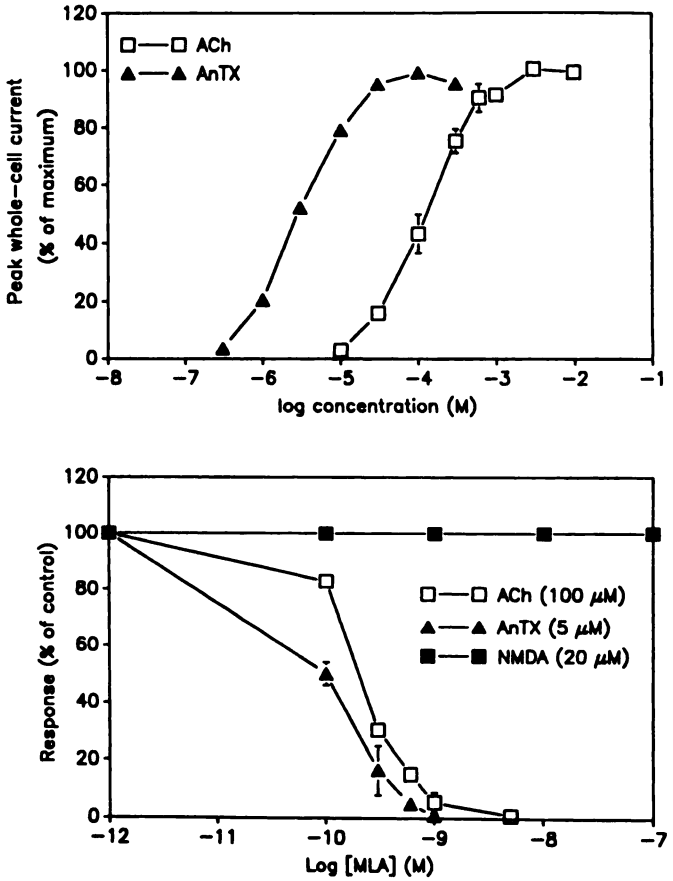

Fig. 3. *Top*, concentration-response curve for the nicotinic agonists ACh and AnTX. The rundown problems were minimized by collecting data at 10 min or more after the patch was established and by using a sequence of low to high concentrations of the agonist in each of the neurons. At the end of the series of agonist applications the low agonist pulse was repeated, and if the response to this concentration changed by >15% from the initial level the cells were discarded. The peak whole-cell currents were normalized with respect to the maximal current evoked by each agonist. Fetal hippocampal neurons grown in culture for 21–35 days were used. Holding potential, −50 mV. *Symbols*, mean values obtained from two neurons in the case of AnTX and mean ± standard error values obtained from five neurons in case of ACh. *Bottom*, concentration-dependent inhibition by MLA of whole-cell currents evoked by 100 μM ACh, 5 μM AnTX, and 20 μM NMDA plus 1 μM glycine. Holding potential, −50 mV. In each neuron, after the control responses were obtained the agonist pulse was repeated under continuous perfusion with MLA (the lowest concentration first), for a period of 10–12 min, which was followed by washing with normal external solution for at least 15 min. At this time, the agonist response of the cell was sampled once more. In stable preparations, the next concentration of MLA was applied, followed by wash and so on. In the most successful cases, three concentrations of MLA could be tested on the same neuron. The peak currents in the presence of MLA were compared with respect to pre-MLA and wash-phase currents, to account for the normal rundown of the response. The number of neurons studied was seven for ACh, three for AnTX, and two for NMDA, and the cells were grown in culture for 20–40 days. The IC_{50} values for MLA, as determined from Hill plots using the average values shown in this figure, were 110 pM and 204 pM for AnTX and ACh, respectively.

The nature of the antagonism exhibited by MLA was further studied at different agonist and antagonist concentrations. Initially, the dose-response relationships for ACh and AnTX were established to select the right range for the agonists in the subsequent studies. AnTX (Fig. 3, *top*) and ACh (Figs. 3, *top*, and 4, *top*) both evoked a concentration-dependent increase in the whole-cell current amplitude. The EC_{50} and the slope values obtained from the Hill plots of these data were 3 μM and 1.31 for AnTX and 126 μM and 1.34 for ACh, respectively. AnTX was found to be 42-fold more potent than ACh on the basis of their EC_{50} values, which is close to earlier estimates (12). The desensitization of the currents seen during a short pulse of either ACh or AnTX was profound at high agonist concentrations. This desensitization could have resulted in an underestimation of the peak amplitude of whole-cell currents at higher concentrations of agonists and may explain the observed slope values (1.31 and 1.34), which are lower than one would expect for the nAChR. A marked concentration-dependent blockade of whole-cell currents evoked by AnTX (5 μM) and ACh (100 μM) was observed when the neurons were perfused with MLA (0.1–1 nM) (Fig. 3, *bottom*). MLA blocked AnTX and ACh responses with comparable IC_{50} values of 110 pM and 204 pM, respectively.

To investigate the mechanism of blockade by MLA, we studied its effect on the various parameters of the whole-cell

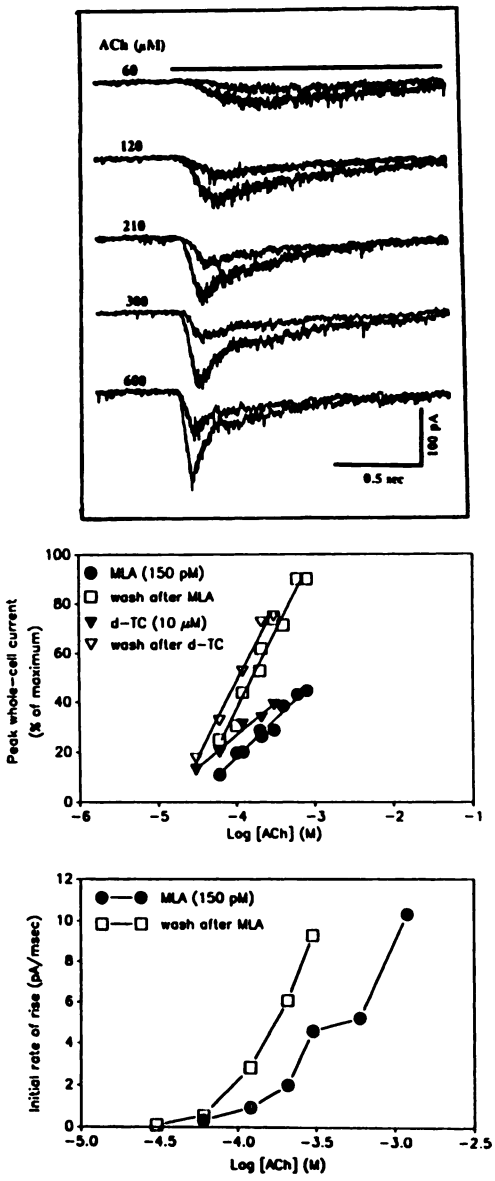

Fig. 4. *Top*, samples of whole-cell current recordings evoked by graded concentrations of ACh in the absence of MLA and after exposure of a single neuron (24 days in culture) to 150 pM MLA. In each pair of traces, the smaller ones were obtained at 12–20 min after perfusion with MLA (i.e., ~20–28 min after the patch was obtained) and the larger ones were obtained after 15–23 min of washing the neuron with normal external solution (i.e., ~43–51 min after the patch). Holding potential, −50 mV. *Middle*, concentration-response curve for ACh after perfusion of the neurons either with MLA (150 pM) for 12 min or with *d*-TC (10 μM) for 12 min, followed by a wash with normal external solution for at least 15 min in each case (protocol same as above). Holding potential, −50 mV. Fetal hippocampal neurons grown for 22–24 days were used. The number of neurons was two for MLA and one for *d*-TC. The currents obtained during the wash phase with 300 μM ACh were estimated to be 75% of the maximal response (in the *d*-TC experiment) and with 600 μM ACh, about 90% of the maximal response (in the MLA experiment), based upon the control dose-response curve shown in Fig. 3, *top*. The data points in the MLA or *d*-TC groups were normalized with respect to the maximum response obtained during the wash phase. A similar pattern of nonparallel shift in the dose-response curves of ACh was seen in another three neurons (data not included here). *Bottom*, concentration-response curve for ACh after perfusion of another neuron (54 days in culture) with 150 pM MLA, using a protocol similar to that described above. In this experiment, MLA (150 pM) was also included in the agonist solutions, as indicated. Initial rate of rise was calculated by dividing the currents evoked by ACh at concentrations that fell in the linear segment of the dose-response curve. Fig. 4 (*top*) shows a typical experiment in which MLA inhibition can be seen at a wide range of agonist concentrations. The plot of the logarithm of agonist concentrations versus normalized peak amplitudes of the whole-cell currents indicates a shift to the right in the curve for ACh in the presence of 150 pM MLA (Fig. 4, *middle*), but in a nonparallel manner. Also, the maximal response to ACh could not be attained by increasing the agonist concentration further (data not shown). Even though these results are suggestive of a noncompetitive mode of action for MLA, such as slow open channel blockade or a change in the agonist-induced desensitization, a competitive action cannot be ruled out. It is unlikely that MLA is an open channel blocker, because this toxin was a more effective antagonist when applied before the agonist than as an admixture with the agonist. If one assumes that MLA blocks the nicotinic currents via enhancement of agonist-induced desensitization, one would expect a significant increase in the decay rate of the currents. However, such an effect was not observed under our experimental conditions (see Fig. 4, *top*). This does not preclude the possibility that MLA causes an agonist-induced desensitization that is too rapid to be detected in the decay phase of the currents.

The question remains of how a putative competitive blocking agent could shift the concentration-response curve of the agonist to the right in a nonparallel manner and decrease the maximal response. This condition could occur if one assumes that MLA dissociates slowly from its binding site on the nAChR, such that the rate of agonist-induced desensitization overcomes the rate of dissociation of the toxin from the receptor. Different indirect approaches have been taken to test this assumption. The first was to verify whether a reversible competitive agent would behave in a manner similar to that of MLA. To this end, we applied *d*-TC (10 μM), via the bathing solution, before testing a pulse of ACh. The whole-cell currents evoked by ACh had a slow rise time after *d*-TC (data not shown), compared with control, which could be predicted for a rapidly reversible competitive blocker. However, the concentration-response curve of ACh was shifted to the right by *d*-TC in a nonparallel manner, an unexpected phenomenon for a competitive blocking agent. Assuming that the reported open channel blocking action of *d*-TC (21, 22) was not a confounding factor in these experiments, these results indicate that the peak whole-cell current measurement may not be an ideal indicator to illustrate the competitive nature of an antagonist, using the rapidly desensitizing nicotinic whole-cell current of hippocampal neurons. Therefore, in the second approach, we used the initial rate of rise of the currents instead of the peak whole-cell currents to quantify the data, because in this method there is less interference from agonist-induced desensitization. The results obtained in this manner showed that MLA was able to shift the concentration-response curve of ACh to the right in a parallel manner (Fig. 4, *bottom*), which is compatible with a competitive mode of action.

The antagonism by MLA of ACh-evoked whole-cell currents was voltage independent (Fig. 5) over the range of −100 to +80 mV, suggesting further that MLA does not interact with sites

fit amplitude by the time constant of the rising phase. The rising phase of the current was fit to a single exponential function using the CLAMPFIT program (pCLAMP). Further details of the calculation of the initial rate of rise will be published elsewhere.

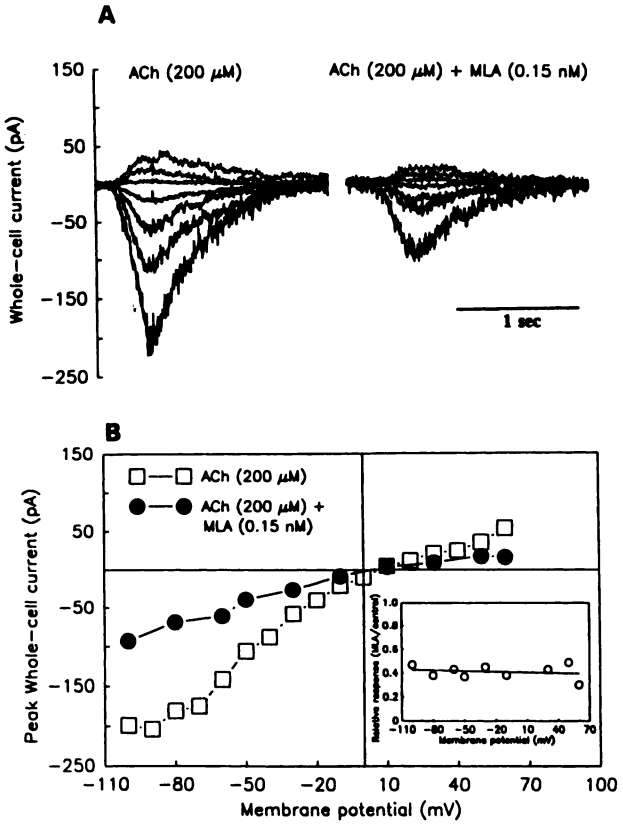

Fig. 5. A, Representative samples of whole-cell currents evoked by ACh (200 μM) at different holding potentials in the absence (*left*) and in the presence of MLA (150 pM) (*right*). Recordings were obtained from a fetal rat hippocampal neuron grown in culture for 24 days. Holding potentials, −100, −50, −30, −10, 10, 30, and 50 mV (*bottom* to *top*, respectively). B, Current-voltage relationship of ACh-evoked whole-cell current peaks in the absence and in the presence of 150 pM MLA. The data were obtained from the same neuron as in A. *Inset*, the peak current obtained in the presence of MLA, normalized with respect to control. The effect of MLA was not voltage dependent. Similar results were observed in another two experiments.

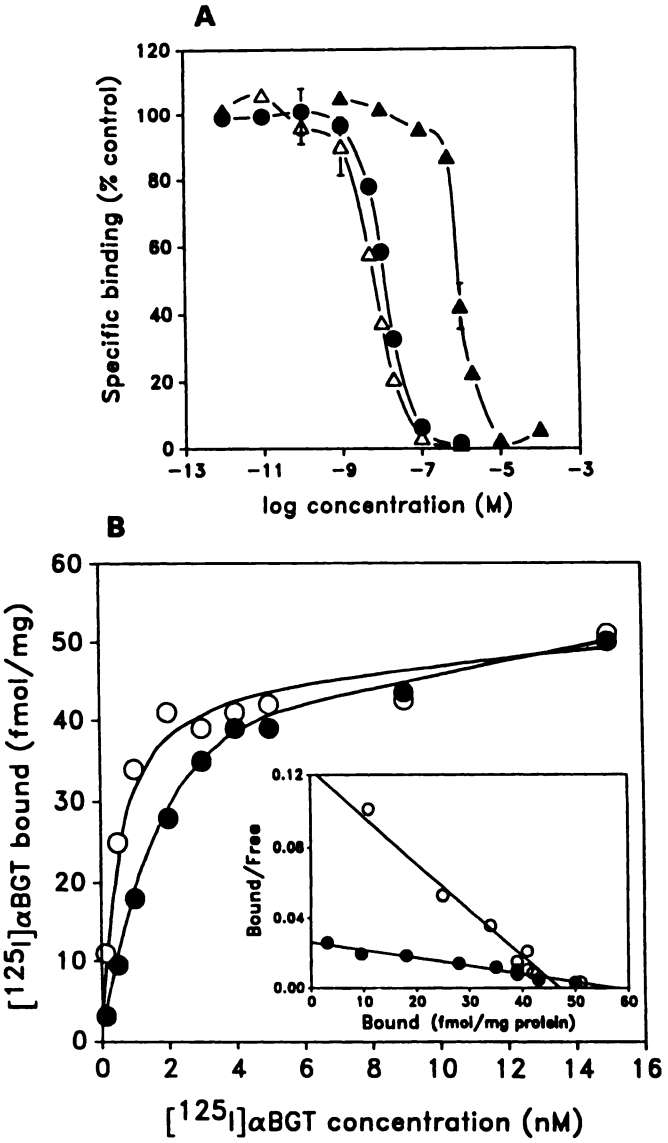

Fig. 6. A, Nicotinic ligand interaction in adult rat hippocampal membranes. Serial dilutions of α-cobratoxin (△), MLA (●), and AnTX (▲) were compared for their abilities to inhibit the binding of ^{125}I-α-BGT (1 nM) to adult rat hippocampal membranes. Results are the mean ± standard error of duplicate independent assays. IC_{50} values (derived by linear regression of Hill plots) were 6.5 nM, 13 nM, and 0.96 μM for α-cobratoxin, MLA, and AnTX, respectively. B, Representative saturation binding assays for ^{125}I-α-BGT binding to adult rat hippocampal membranes in the absence (○) and presence (●) of 10 nM MLA. *Inset*, Scatchard analysis of the curves shown in B.

located inside the ion channel. Similar voltage-independent action for MLA has been found at the neuronal nAChR of the cockroach *Periplaneta americana* (23). The ability of MLA to inhibit ^{125}I-α-BGT binding to adult rat hippocampal membranes (Fig. 6A) (K_i = 4.3 nM) is also consistent with a competitive mode of action. MLA was equipotent with α-cobratoxin (K_i = 2.5 nM) and 2 orders of magnitude more potent than the agonist AnTX (K_i = 0.32 μM). Compared with its blockade of agonist-evoked whole-cell currents, MLA is more than 1 order of magnitude less potent in the binding assay. This discrepancy is likely to reflect technical differences, as well as differences in the tissue preparations used. There is precedent for subtle differences between adult and fetal forms of nAChR in muscle (24), and similar developmental changes may also occur in neurons. Saturation binding assays for ^{125}I-α-BGT binding to hippocampal membranes were carried out in the presence and in the absence of MLA, at a concentration (10 nM) approximating its IC_{50} value in the competition binding assay. MLA shifted the binding curve to the right, without depressing the plateau value (Fig. 6B), and this is clearly demonstrated in the Scatchard plot (Fig. 6B, *inset*). The maximal number of ^{125}I-α-BGT binding sites, was unchanged (B_{max} control, 51.1 ± 4.7; MLA, 55.3 ± 1.2 fmol/mg of protein), whereas the affinity for the snake toxin was decreased in the presence of MLA (K_d, control, 0.54 ± 0.18 nM; in the presence of MLA, 1.89 ± 0.22 nM).

To determine whether this competitive interaction is relevant to the functional blockade achieved by MLA, we evaluated whether MLA could protect against the long-lasting inhibition of agonist-evoked whole-cell currents induced by α-BGT in hippocampal neurons (12). Preincubation of the cultures with 1 μM α-BGT for 60 min resulted in a significant decrease in the sensitivity of the neurons to AnTX, when tested during the subsequent 2-hr wash period. For instance, mean ± standard error peak current evoked by 10 μM AnTX under control conditions was 161 ± 37 pA (n = 7 neurons), whereas that

obtained after incubation with 1 μM α-BGT was 12 ± 2 pA (n = 14 neurons), indicating that the blockade caused by this toxin was not reversed during the wash phase. On the other hand, when the cultures were exposed to 1 nM MLA for 75 min and then washed with normal external solution for 15 min before the experiment was conducted, we obtained a mean current of 108 ± 44 pA (n = 4 neurons), which was almost indistinguishable from that of the control group, as would be expected if the MLA effect was completely reversible (Fig. 1). However, when the cultures were exposed to 1 nM MLA for 15 min before α-BGT, 60 min together with α-BGT, and 15 min after the combination, followed by a 15-min wash with normal external solution, there was a significant recovery of the AnTX response during the wash phase; the mean peak current of 102 ± 41 pA (n = 8 neurons) was comparable to that observed after preincubation with MLA alone. These results suggest that MLA and α-BGT have a common site of action on the nAChR of hippocampal neurons, such that the reversible MLA could protect the receptor from an irreversible blockade caused by α-BGT.

Finally, the effect of MLA was tested on the nicotinic single-channel currents recorded from fetal hippocampal neurons, using outside-out patch-clamp conditions. At concentrations between 1 and 1000 fM, MLA decreased significantly ($p < 0.001$) the frequency of channel openings (Fig. 7) activated by 1 μM AnTX in outside-out patches obtained from these neurons. The blocking effect and the recovery were readily observed when the patch pipette was immersed in MLA-containing and MLA-free external solutions, respectively. On the other hand, MLA seemed to have no effect on the mean channel open time or on the main single-channel conductance. This suggests that MLA inhibits the nAChR in the closed conformation, which is also consistent with a competitive action. The concentration of MLA required to block the nAChR was much lower in single-channel experiments than in whole-cell current experiments. One possible explanation is the small number of receptors in an outside-out patch, particularly in the case of nAChR of hippocampal neurons. It is interesting to note that a similar concentration difference between the two techniques was observed with another agent, d-TC. At 1–10 nM d-TC, there was a 35–75% decrease in the single-channel opening frequency, whereas about 50% blockade of the whole-cell peak currents was observed with 10 μM, a difference of 3–4 orders of magnitude.

MLA displayed remarkably high affinity and potency, in both ligand binding assays and functional studies, being comparable to or better than the snake α-toxins, but the action of MLA was reversible, like that of curarimimetic agents. Neuronal nAChR are heterogeneous (3), and distinct subtypes can be discerned by high affinity [^{3}H]nicotine, neuronal (or κ-) BGT, and α-BGT binding. [^{3}H]Nicotine sites have been tentatively correlated with presynaptic nAChR mediating dopamine release in rat striatum (25), whereas neuronal BGT may distinguish the subtype that predominates in autonomic ganglia (26). Both of these preparations are relatively insensitive to MLA, with IC_{50} values for the toxin of about 5 μM.[1] (27). More surprising is the similar insensitivity of muscle nAChR to MLA (15). This was confirmed in single-channel recordings obtained from outside-out patches excised from fetal rat myoballs grown in culture. An MLA concentration of 1 μM was required to block fully AnTX-induced single-channel currents recorded from myoballs (data not shown), whereas the sensitivity of

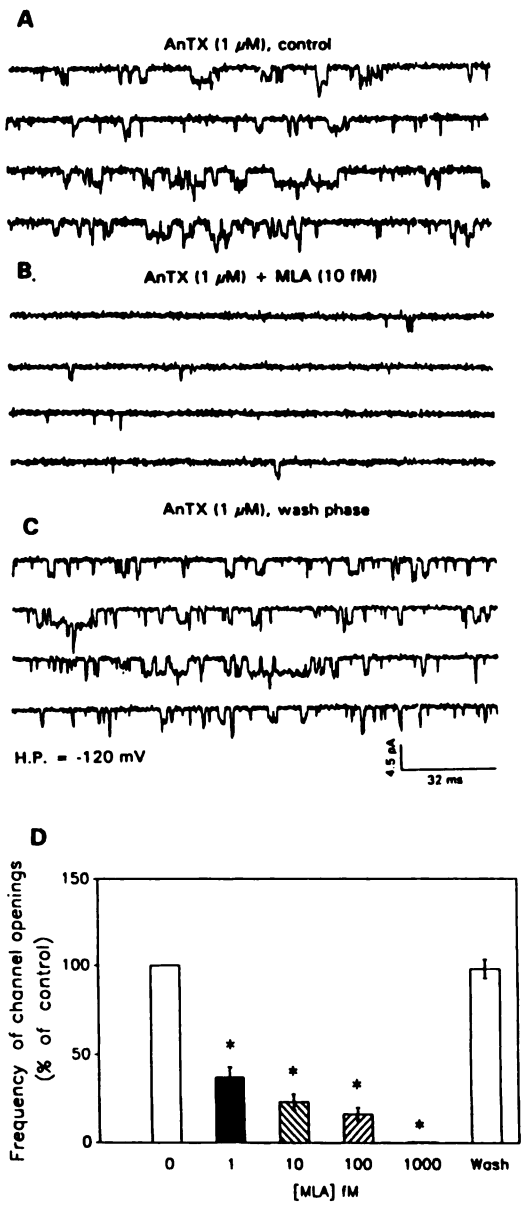

Fig. 7. Samples of single-channel currents evoked by AnTX (1 μM) alone or in the presence of MLA. Recordings were made from an outside-out patch obtained from a fetal rat hippocampal neuron. A, Control condition; B, in the presence of 10 fM MLA; C, during wash phase; D, summary of the effect of different concentrations of MLA (1–1000 fM) on the frequency of channel openings induced by AnTX, normalized with respect to the control condition. Each *bar* represents mean ± standard error obtained from three different neurons (age of culture = 12–15 days). *, $p < 0.001$.

nAChR on hippocampal neurons was 6 orders of magnitude higher. These results establish that MLA is more subtype selective than any other nicotinic antagonist thus far identified. This striking discrimination is paralleled by the relative binding affinities of MLA for the agonist/competitive antagonist site of the respective AChR (6, 16). Presumably, the unusual preference of MLA for neuronal α-BGT-sensitive nAChR reflects the unique genes (α_7 and α_8) proposed to encode this receptor in the brain (28, 29). The gene cloning of this nAChR subtype has confirmed that it is distinct from the muscle nAChR, despite the shared sensitivity to snake α-neurotoxins. The failure to demonstrate a nicotinic function of α-BGT-sensitive

nAChR in the brain may reflect, in part, the lack of appropriate tools and a reliance on α-BGT; the large size, the slowness of action, and the pseudoirreversible nature of α-BGT limit its utility. MLA, as a small, reversible, and potent antagonist, may help to unveil the mystery of brain α-BGT-sensitive nAChR (30). Although most of the results in this study suggest that the novel neurotoxin MLA may have a competitive antagonist interaction at the nAChR of the hippocampal neurons, the limitations of some of the techniques do not permit us to conclude unequivocally that MLA is acting solely by this mechanism. Improved techniques such as very rapid solution exchange in the whole-cell experiments and the possible use of radiolabeled MLA are some of the possible directions for future investigation into the mechanism of action of this toxin.

Acknowledgments

We thank Ms. Mabel A. Zelle for technical assistance and for helpful comments and Mrs. Barbara Marrow for technical assistance.

References

1. Changeux, J.-P. Functional architecture and dynamics of the nicotinic acetylcholine receptor: an allosteric ligand-gated ion channel. *Fidia Res. Found. Neurosci. Award Lect.* **4**:21–168 (1990).
2. Albuquerque, E. X., J. W. Daly, and J. E. Warnick. Macromolecular sites for specific neurotoxins and drugs on chemosensitive synapses and electrical excitation in biological membranes, in *Ion Channels* (T. Narahashi, ed.), Vol. 1. Plenum Press, New York, 95–162 (1988).
3. Deneris, E. S., J. Connolly, S. W. Rogers, and R. Duvoisin. Pharmacological and functional diversity of neuronal nicotinic acetylcholine receptors. *Trends Pharmacol. Sci.* **12**:34–40 (1991).
4. Clark, P. B. S., R. D. Schwartz, S. M. Paul, C. D. Pert, and A. Pert. Nicotinic binding in rat brain: autoradiographic comparison of [^{3}H]acetylcholine, [^{3}H] nicotine, and [^{125}I]α-bungarotoxin. *J. Neurosci.* **5**:1307–1315 (1985).
5. Marks, M. J., J. A. Stitzel, E. Romm, J. M. Weher, and A. C. Collins. Nicotinic binding sites in the rat and mouse brain: comparison of acetylcholine, nicotine and α-bungarotoxin. *Mol. Pharmacol.* **30**:427–436 (1986).
6. Macallan, D. R. E., G. G. Lunt, S. Wonnacott, K. L. Swanson, H. Rapoport, and E. X. Albuquerque. Methyllycaconitine and (+)-anatoxin-a differentiate between nicotinic receptors in vertebrate and invertebrate nervous systems. *FEBS Lett.* **226**:357–363 (1988).
7. Pabreza, L. A., S. Dhawan, and K. J. Kellar. [^{3}H]Cytisine binding to nicotinic cholinergic receptors in brain. *Mol. Pharmacol.* **39**:9–12 (1991).
8. Lipton, S. A., E. Aizenman, and R. H. Loring. Neural nicotinic acetylcholine responses in solitary mammalian retinal ganglion cells. *Pflügers Arch.* **410**:37–43 (1987).
9. Zhang, Z. W., and P. Feltz. Nicotinic acetylcholine receptors in porcine hypophyseal intermediate lobe cells. *J. Physiol. (Lond.)* **422**:83–101 (1990).
10. Mulle, C., and J.-P. Changeux. A novel type of nicotinic receptor in the rat central nervous system characterized by patch-clamp technique. *J. Neurosci.* **10**:169–175 (1990).
11. Alkondon, M., and E. X. Albuquerque. α-Cobratoxin blocks the nicotinic acetylcholine receptor in rat hippocampal neurons. *Eur. J. Pharmacol.* **191**:505–506 (1990).
12. Alkondon, M., and E. X. Albuquerque. Initial characterization of the nicotinic acetylcholine receptors in rat hippocampal neurons. *J. Receptor Res.* **11**:1001–1022 (1991).
13. Aracava, Y., S. S. Deshpande, K. L. Swanson, H. Rapoport, S. Wonnacott, G. Lunt, and E. X. Albuquerque. Nicotinic acetylcholine receptors in cultured neurons from the hippocampus and brain stem of the rat characterized by single channel recording. *FEBS Lett.* **222**:63–70 (1987).
14. de la Garza, R., T. J. McGuire, R. Freedman, and B. J. Hoffer. Selective antagonism of nicotinic actions in the rat cerebellum with α-bungarotoxin. *Neuroscience* **23**:887–891 (1987).
15. Jennings, K. R., D. G. Brown, and D. P. Wright, Jr. Methyllycaconitine, a naturally occurring insecticide with a high affinity for the insect cholinergic receptor. *Experientia (Basel)* **42**:611–613 (1986).
16. Nambi-Aiyar, V., M. H. Benn, T. Hanna, J. Jacyno, S. H. Roth, and J. L. Wilkens. The principal toxin of *Delphinium brownii* (*Rydb.*) and its mode of action. *Experientia (Basel)* **35**:1367–1368 (1979).
17. Ward, J. M., V. B. Cockcroft, G. G. Lunt, F. S. Smillie, and S. Wonnacott. Methyllycaconitine: a selective probe for neuronal α-bungarotoxin binding sites. *FEBS Lett.* **270**:45–48 (1990).
18. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**:85–100 (1981).
19. Albuquerque, E. X., A. C. S. Costa, M. Alkondon, K. P. Shaw, A. S. Ramoa, and Y. Aracava. Functional properties of the nicotinic and glutamatergic receptors. *J. Receptor Res.* **11**:603–625 (1991).
20. Vyklický, L., Jr., M. Benveniste, and M. L. Mayer. Modulation of *N*-methyl-D-aspartic acid receptor desensitization by glycine in mouse cultured hippocampal neurons. *J. Physiol. (Lond.)* **428**:313–331 (1990).
21. Ascher, P., A. Marty, and T. O. Neild. The mode of action of antagonists of the excitatory response to acetylcholine in *Aplysia* neurons. *J. Physiol. (Lond.)* **278**:207–235 (1978).
22. Shaker, N., T. Eldefrawi, L. G. Aguayo, J. E. Warnick, and E. X. Albuquerque. Interactions of *d*-tubocurarine with the nicotinic acetylcholine receptor/channel molecule. *J. Pharmacol. Exp. Ther.* **220**:172–177 (1982).
23. Sattelle, D. B., R. D. Pinnock, and S. C. R. Lummis. Voltage-independent block of a neuronal nicotinic acetylcholine receptor by *N*-methyllycaconitine. *J. Exp. Biol.* **142**:215–224 (1989).
24. Mishina, M., T. Takai, K. Imoto, M. Noda, T. Takahashi, S. Numa, C. Methfessel, and B. Sakmann. Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature (Lond.)* **321**:406–411 (1986).
25. Rapier, C., G. G. Lunt, and S. Wonnacott. Nicotinic modulation of [^{3}H] dopamine release from striatal synaptosomes: pharmacological characterisation. *J. Neurochem.* **54**:937–945 (1990).
26. Berg, D. K., R. T. Boyd, S. W. Halvorsen, L. S. Higgins, M. H. Jacob, and J. F. Margiotta. Regulating the number and function of neuronal acetylcholine receptors. *Trends Neurosci.* **12**:16–21 (1989).
27. Drasdo, A., M. Caulfield, D. Bertrand, S. Bertrand, and S. Wonnacott. Methyllycaconitine, a novel nicotinic antagonist. *Mol. Cell. Neurosci.* (In press).
28. Schoepfer, R., W. G. Conroy, P. Whiting, M. Gore, and J. Lindstrom. Brain α-bungarotoxin binding protein cDNAs and MAbs reveal subtypes of this brand of the ligand-gated ion channel superfamily. *Neuron* **5**:35–48 (1990).
29. Couturier, S., D. Bertrand, J.-M. Matter, M.-C. Hernandez, S. Bertrand, N. Millar, S. Valera, T. Barkas, and M. Ballivet. A neuronal nicotinic acetylcholine receptor subunit (α7) is developmentally regulated and forms a homo-oligomeric channel blocked by α-BTX. *Neuron* **5**:847–856 (1990).
30. Wonnacott, S., E. X. Albuquerque, and D. Bertrand. Methyllycaconitine: a selective probe for neuronal αbungarotoxin binding sites. *Methods Neurosci.* (In press).

Send reprint requests to: Edson X. Albuquerque, Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, 655 West Baltimore St., Baltimore, MD 21201–1559.