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Potentiation of the actions of acetylcholine, epibatidine, and nicotine by methyllycaconitine at fetal muscle-type nicotinic acetylcholine receptors

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ABSTRACT

Methyllycaconitine (MLA) is a norditerpenoid alkaloid found in high abundance in toxic *Delphinium* (larkspur) species. It is a potent and selective antagonist of α_7-nicotinic acetylcholine receptors, but has not been well investigated for activity aside from receptor antagonism. The aim of this study was to investigate the effects of MLA alone and in combination with acetylcholine, epibatidine, nicotine, and neostigmine for actions other than receptor antagonism in TE-671 cells expressing $(\alpha_1)_2\beta_1\gamma\delta$ nicotinic acetylcholine receptors. Ligand activity was assessed through measurements of membrane potential changes in TE-671 cells using a fluorescent membrane potential-sensitive dye and normalized to the maximum response to epibatidine (10 μM). MLA was ineffective in changing cell membrane potential in the absence of other receptor agonists. However at nanomolar concentrations, it acted as a co-agonist to potentiate TE-671 cell responses to acetylcholine, epibatidine, nicotine, and neostigmine. These results suggest that the poisoning of cattle by norditerpenoid alkaloids found in larkspur may be more complex than previously determined.

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1. Introduction

Fetal muscle-type nicotinic acetylcholine receptors are ligand-gated cation channels composed of $(\alpha_1)_2\beta_1\gamma\delta$ subunits with ligand binding sites located at the interfaces of the $\alpha\delta$ and $\alpha\gamma$ subunits and are activated by two molecules of agonist (Arias, 1997, 2000; Blount and Merlie, 1989). Ligands that bind to these receptors can act as agonists, antagonists, allosteric modulators, or co-agonists that can potentiate the response of these receptors to low concentrations of agonist (Cachelin and Rust, 1994; Smulders et al., 2005). For example, (d)-tubocurarine acting as a co-agonist can potentiate the acetylcholine response in the rat parasympathetic ganglia (Ascher et al., 1979). (d)-tubocurarine can potentiate the response of $\alpha_2\beta_4$ and $\alpha_3\beta_4$ nicotinic acetylcholine receptors expressed by *Xenopus* oocytes to acetylcholine (Cachelin and Rust, 1994), and potentiate acetylcholine action at fetal muscle-type nicotinic acetylcholine receptors expressed by quail fibroblasts (Steinbach and Chen, 1995). Cachelin and Rust (1994) proposed that the potentiation of these receptors was caused by the binding of one molecule of (d)-tubocurarine and one molecule of agonist to the receptor α subunits. This has also been described as a "heteroliganded" receptor state by Steinbach and Chen (1995). Other drugs such as atropine and acetylcholinesterase inhibitors act in a similar manner and have been termed co-agonists (Smulders et al., 2005; Zwart et al., 2000; Zwart and Vijverberg, 1997).

One naturally-occurring ligand that has not been investigated as a co-agonist is MLA. MLA is found in toxic larkspur (*Delphinium* spp.) and is a potent and selective antagonist of α_7-nicotinic acetylcholine receptors. MLA induces single channel currents from *Xenopus* oocytes expressing rat fetal muscle-type nicotinic acetylcholine receptors; its action is sensitive to α-bungarotoxin (Cooper et al., 1996). The affinity value of MLA is in the nanomolar concentration range at α_7-nicotinic acetylcholine receptors but three orders of magnitude higher at muscle-type, $\alpha_4\beta_2$ and $\alpha_3\beta_4$- nicotinic acetylcholine receptors (Sharples and Wonnacott, 2001). The K_i of MLA in inhibiting [^{125}I]α-bungarotoxin binding to TE-671 cells is 63 ± 14 μM (Ward et al., 1990).

MLA is of agricultural importance as a cattle toxicant. Cattle grazing in mountain pastures with larkspur containing high concentrations of MLA repeatedly cycle between one or two days of high consumption of larkspur followed by a period of detoxification (Pfister et al., 1997a, 1997b). If cattle consume large amounts of larkspur too quickly, they exhibit muscular weakness and lateral recumbency often leading to death. It is possible that in addition to its "curariform-like" effects (Benn and Jacyno, 1983), MLA acts in some other manner in cattle to facilitate cyclic larkspur consumption.

The aim of this study was to investigate the effects of MLA alone and in combination with acetylcholine, epibatidine, nicotine, and neostigmine for actions other than nicotinic cholinergic receptor antagonism. Ligand activity was assessed through nicotinic cholinergic receptor-induced changes in membrane potential in TE-671 rhabdomyosarcoma cells. Our results indicate that MLA alone has no intrinsic efficacy in TE-671 cells but that it can act as a co-agonist to

Abbreviation: MLA, Methyllycaconitine.

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potentiate cellular electrical responses to three nicotinic acetylcholine receptor agonists and the anticholinesterase agent neostigmine.

2. Material and methods

Racemic epibatidine, (d)-tubocurarine chloride hydrate, neostigmine methylsulfate, and acetylcholine chloride were obtained from Sigma-Aldrich (St. Louis, MO). (−)-Nicotine ditartrate was obtained from Calbiochem (EMD Biosciences, La Jolla, CA). Fetal bovine serum and penicillin/streptomycin were from Media Tech, Inc. (Herndon, VA). Dulbecco's modified Eagle's medium was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The fluorescence dye kits were purchased from Molecular Devices (Sunnyvale, CA). MLA and lycoctonine were isolated from larkspur and purified as previously described (Manners et al., 1991; Manners et al., 1993).

2.1. Nicotinic agonist activity at human fetal nicotinic acetylcholine receptors

The rhabdomyosarcoma cell line TE-671 was obtained from ATCC (Manassas, VA, USA). Membrane depolarization produced by nicotinic agonists was measured by changes in fluorescence of a membrane potential-sensitive dye as previously described (Green et al., 2010). KCl (40 mM) was used as a depolarizing calibrant to control for differences of dye loading and cell count between wells as described by Lee et al. (2006). Receptor ligand or KCl additions and membrane potential measurements were performed using a Flexstation II (Molecular Devices Corporation, Sunnyvale, CA, USA). Readings were taken every 1.12 s for 255 s, for a total of 228 electrical measurements per well. In control experiments involving the addition of a receptor ligand and KCl, the first 17 s were used for a baseline reading; at 18 s, 50 μL of a test compound were added to assess agonist activity; and at 180 s, 25 μL of KCl in dye-saline solution were added to attain a final extracellular concentration of 40 mM K^+. For experiments involving the addition of two receptor ligands and KCl, serial dilutions of an antagonist followed by single concentrations of an agonist were made. This method allowed for the direct comparison of concentration-dependent antagonist effects within each 96 well plate. The timing of the compound additions was as follows. At 18 s, 30 μL of antagonist (MLA or (d)-tubocurarine) were added; at 98 s, 30 μL of agonist were added; and at 180 s, 25 μL of KCl were added. An 80 s incubation period after the addition of antagonist was used to control for the change in dye fluorescence due to addition of drug solutions, and to detect any changes in membrane potential. We corrected for the response of the dye solution to the addition of fluid in the three-compound addition experiments by measuring the maximum agonist response from 70 to 180 s and the maximum KCl response from 70 to 260 s and required a stable baseline fluorescent response. An assessment of the cellular electrical response to 10 μM epibatidine was included in every experiment as an internal control and dye only solution was added at 18 s, and epibatidine was added at 98 s. We chose this concentration based on previous work with TE-671 cells, which has shown that 10 μM epibatidine produces a maximal increase in membrane potential (Green et al., 2010).

2.2. Thirty-minute MLA preincubation protocol

Serial dilutions of MLA were preloaded in 10-fold increments for final concentrations of 1 pM to 100 μM into the TE-671 cell assay plate with Molecular Devices Membrane Potential Blue Dye. The TE-671 cells in the presence of the MLA-dye solution were rested in the dark for 30 min prior to reading on Flexstation II (Akos Nemecz, University of California San Diego, Personal communication, Experimental Biology 2010, Anaheim, CA). The acetylcholine control and epibatidine control concentration-effect curve wells contained the same final volume of membrane potential sensing dye as the MLA containing wells. Plate reading and data analysis were identical to the two compound addition experiments described above.

2.3. Data analysis

Depolarizing responses to agonists were normalized to the corresponding maximum epibatidine response for each respective plate, fitted to a sigmoidal dose-response equation, and graphed with Prism version 4.03 (GraphPad Software, San Diego, CA, USA) to determine EC_{50} using a sigmoidal dose-response equation. Data are displayed as a percentage of the maximal epibatidine response (Lee et al., 2008). Comparisons of the logEC_{50} values for the concentration-effect curves were made by F test. If there were significant differences between EC_{50} values, further comparisons of the variation between agonist and MLA concentrations were done with a two-way analysis of variance followed by a Bonferroni post test. Comparisons of multiple treatment means versus control values were performed using a one-way analysis of variance followed by Tukey's multiple comparison test. In all cases, the limit for statistical significance was set at $P<0.05$.

3. Results

3.1. Actions of MLA alone in TE-671 cells

Initially, a two-compound addition experiment was conducted to test if MLA possessed any intrinsic efficacy at $(\alpha_1)_2\beta_1\gamma\delta$ receptors expressed by TE-671 cells. As shown in Fig. 1, MLA was without effect in TE-671 cells when added at concentrations ranging from 35 nM to 100 μM. Moreover, there were no significant differences between the cellular responses of TE-671 cells to any concentration of MLA tested ($P=0.5339$, ANOVA, $n=8$ concentrations of MLA). There was a slight change in baseline fluorescence after addition of MLA at any concentration to a well due to minor dye dilution (Fig. 1 inset).

3.2. Rank order of agonist potency in TE-671 cells

Concentration effect curves for acetylcholine, epibatidine, nicotine and neostigmine were generated in two compound addition experiments and are displayed as the control responses in Figs. 2–6A. The nicotinic agonist rank order of potency in TE-671 cells was determined to be epibatidine>acetylcholine>nicotine>neostigmine

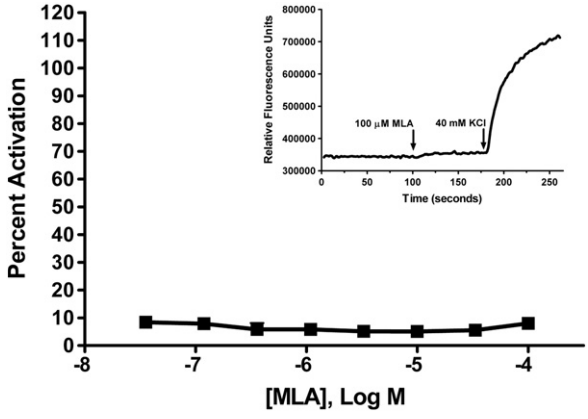

Fig. 1. The concentration-effect relationship and representative tracing for the actions of MLA on membrane potential sensing dye fluorescence in TE-671 cells. In each experiment, the membrane depolarization resulting from the addition of MLA in $\log_{10}$ molar concentrations was measured and displayed as a percentage of the 10 μM epibatidine response. Each datum point represents the mean ± S.E.M. of three experiments of duplicate wells. The inset figure is a representative tracing of a membrane potential sensing dye response of a single experiment from a single well.

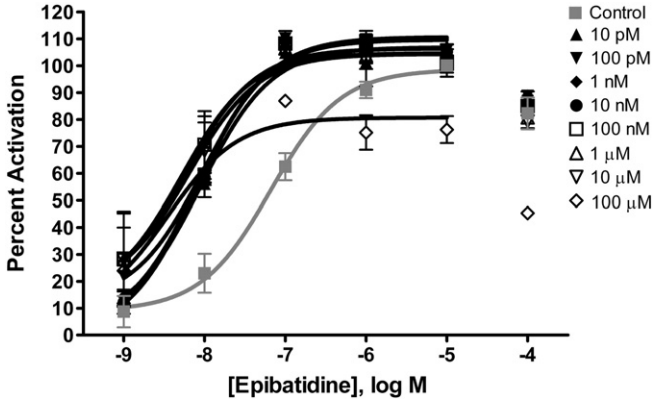

Fig. 2. The actions of MLA on varying concentrations of epibatidine on membrane potential sensing dye response in TE-671 cells. The membrane depolarization resulting from the addition of epibatidine in $\log_{10}$ molar concentrations was measured and displayed as a percentage of the of the 10 μM epibatidine response for each respective 96 well plate. Each datum point represents the mean ± S.E.M. of three to five experiments of duplicate wells for MLA pretreatment. Each datum point of the control epibatidine concentration-effect curve represents 30 independent experiments of duplicate wells.

(EC_{50} values for epibatidine, acetylcholine, and nicotine were 66.2 nM, 400.4 nM, 4.1 μM, respectively). Epibatidine at a concentration of 10 μM elicited the maximum change in membrane potential sensing dye fluorescence. Based on this observation we used 10 μM epibatidine as the reference response in subsequent three-compound addition experiments to which all other responses to receptor ligands were normalized in individual 96 well plates.

3.3. MLA potentiation of receptor agonist responses

For the three-compound addition experiments with TE-671 cells, varying concentrations of MLA from 10 pM to 100 μM were added prior to the addition of a single concentration of agonist. Epibatidine was the first nicotinic acetylcholine receptor agonist tested for potentiation by MLA using three-compound addition experiments. The epibatidine concentration response curves were shifted to the left and the logEC_{50}s were significantly different from control ($P<0.0001$, F test, n = 8 concentrations of MLA), (Fig. 2, Table 1). Pretreatment with 100 μM MLA was unique in that it significantly increased the TE-671 cell response to low concentrations of epibatidine. One hundred μM MLA also decreased the TE-671 cell response to higher concentrations of epibatidine ($P<0.05$, two-way ANOVA, Bonferroni post test, responses to10 nM epibatidine in the presence and absence of MLA or responses to 100 μM epibatidine in the presence and absence of MLA).

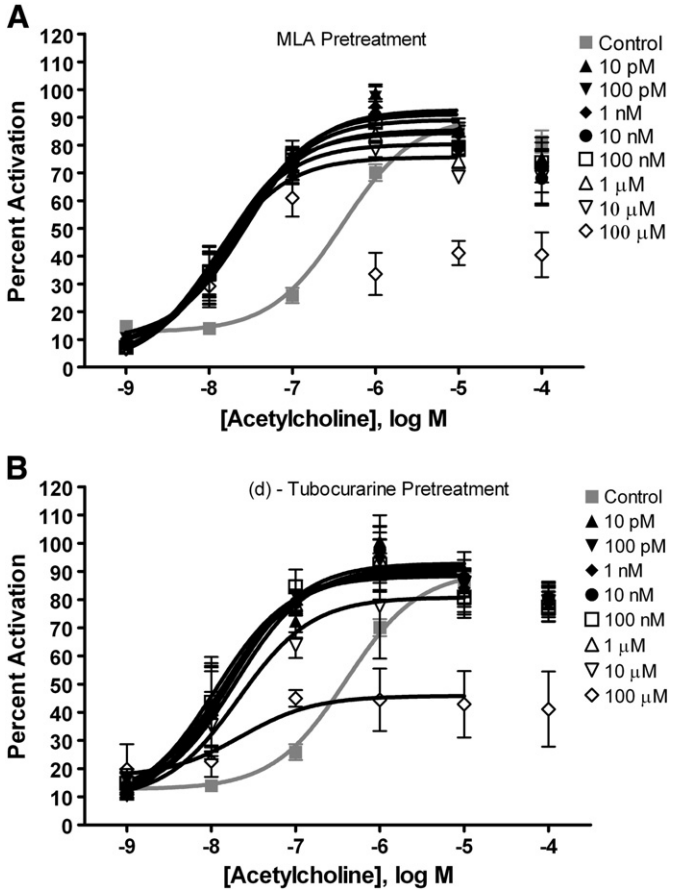

Fig. 3. The actions of MLA (A) and (d)-tubocurarine (B) on varying concentrations of acetylcholine on the membrane potential sensing dye response in TE-671 cells. The membrane depolarization resulting from the addition of acetylcholine in $\log_{10}$ molar concentrations was measured and displayed as a percentage of the of the 10 μM epibatidine response. Each datum point represents the mean ± S.E.M. of four to six experiments of duplicate wells for MLA pretreatment, or three to five experiments of duplicate wells for (d)-tubocurarine pretreatment. Each datum point of the control acetylcholine concentration-effect curve represents the mean ± S.E.M. of 20 independent experiments of duplicate wells and is displayed for reference in both figures.

Acetylcholine, the endogenous ligand for nicotinic acetylcholine receptors, was also tested in combination with MLA in TE-671 cells. The acetylcholine concentration effect curves were shifted to the left

Table 1
Epibatidine concentration-effect curve EC_{50} and percent maximum response values in TE-671 cells pretreated with 100 μM to 10 pM MLA.

Antagonist pretreatment, molar concentration	Epibatidine EC_{50} (nM)	EC_{50} 95% confidence intervals	Percent maximum response, mean ± S.E.M.
Control, epibatidine	66.2	32.5–134.7	100 ± 1[a]
MLA, 10 pM	9.0	4.9–16.8	107 ± 2
MLA, 100 pM	9.7	5.9–16.2	110 ± 3
MLA, 1 nM	8.3	5.9–11.8	109 ± 2
MLA, 10 nM	8.0	5.5–11.6	109 ± 2
MLA, 100 nM	5.9	1.6–22.0	108 ± 1
MLA, 1 μM	5.2	1.3–19.9	106 ± 1
MLA, 10 μM	6.4	1.5–27.3	104 ± 1
MLA, 100 μM	3.5	0.3–36.3	87 ± 12

n = 30 experiments for epibatidine control, 4–5 experiments for MLA concentrations from 10 pM to 1 μM and 3–4 experiments of 10 and 100 μM MLA.
[a] Epibatidine responses were normalized to the maximum epibatidine response for each plate respectively.

Table 2
Acetylcholine concentration-effect curve EC_{50} and percent maximum response values in TE-671 cells pretreated with 100 μM to 10 pM MLA.

Antagonist pretreatment, molar concentration	ACh[a] EC_{50} (nM)	EC_{50} 95% confidence intervals	Percent maximum response, mean ± S.E.M.
Control, ACh	400.4	166.5–962.9	86 ± 3
MLA, 10 pM	28.1	1.8–451.8	99 ± 3
MLA, 100 pM	22.5	1.6–312.2	97 ± 4
MLA, 1 nM	22.0	2.6–186.4	94 ± 3
MLA, 10 nM	17.4	1.7–174.4	91 ± 3
MLA, 100 nM	14.2	2.1–94.7	88 ± 2
MLA, 1 μM	12.6	1.2–136.7	84 ± 2
MLA, 10 μM	11.5	1.1–125.2	79 ± 3
MLA, 100 μM	N.C.[b]	N.C.[b]	N.C.[b]

n = 20 experiments for acetylcholine control and 4–6 experiments for all MLA concentrations.
[a] Acetylcholine.
[b] Not calculated due to poor fit.

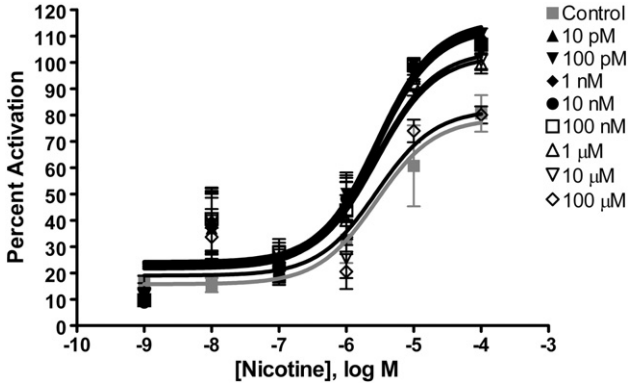

Fig. 4. The actions of MLA on varying concentrations of nicotine on membrane potential sensing dye response in TE-671 cells. The membrane depolarization resulting from the addition of nicotine in $\log_{10}$ molar concentrations was measured and displayed as a percentage of the of the 10 μM epibatidine response. Each datum point represents the mean ± S.E.M. of three to five experiments of duplicate wells for MLA pretreatment. Each datum point of the control nicotine concentration-effect curve represents six independent experiments of duplicate wells.

and the logEC_{50}s were significantly different from control ($P<0.05$, F test, n = 8 concentrations of MLA) (Fig. 3A, Table 2). As was observed with epibatidine, 100 μM MLA significantly increased the TE-671 cell responses to low concentrations of acetylcholine ($P<0.05$, two-way ANOVA, Bonferroni post test, responses to 100 nM acetylcholine in presence and absence of 100 μM MLA). As the concentration of acetylcholine was increased to 1 μM and above, 100 μM MLA significantly decreased the responses of TE-671 cells ($P<0.05$, two-way ANOVA, Bonferroni post test, responses to ≥ 1.0 μM acetylcholine in the presence and absence of MLA).

In MLA-treated TE-671 cells, concentration-effect curves for nicotine were similarly shifted downward and to the left (Fig. 4, Table 3). However, there were no significant differences between the logEC_{50}s ($P = 0.9938$, F test, n = 8 concentrations of MLA). Unlike acetylcholine and epibatidine, nicotine effects on TE-671 cells were neither potentiated by 100 μM MLA at low agonist concentrations nor was the cellular response to nicotine at concentrations ≥ 1.0 μM affected.

As a comparison to the work of other investigators, the actions of (d)-tubocurarine on acetylcholine were also assessed. In the presence of this receptor antagonist the concentration response curves for acetylcholine were shifted to the left. The EC_{50}s were decreased and maximum responses of the cells increased for seven of the eight (d)-tubocurarine concentrations tested (Fig. 3B, Table 4). Moreover, the logEC_{50}s of the concentration-effect curves were significantly different ($P<0.0001$, F test, n = 8 concentrations of (d)-tubocurarine). Concentrations of 10 μM and 100 μM (d)-tubocurarine decreased the maximum response of TE-671 cells to acetylcholine and (d)-tubocurarine at all concentrations examined decreased the TE-671 cell response to 100 μM acetylcholine.

Table 3
Nicotine concentration-effect curve EC_{50} and percent maximum response values in TE-671 cells pretreated with 100 μM to 10 pM MLA.

Antagonist pretreatment, molar concentration	Nicotine EC_{50} (μM)	EC_{50} 95% confidence intervals	Percent maximum response, mean ± S.E.M.
Control, nicotine	4.1	1.1–1.6	81 ± 7
MLA, 10 pM	2.7	1.2–6.4	111 ± 1
MLA, 100 pM	2.2	1.0–5.1	110 ± 3
MLA, 1 nM	2.3	1.0–5.7	109 ± 2
MLA, 10 nM	2.1	0.9–5.0	106 ± 2
MLA, 100 nM	2.7	1.0–7.3	108 ± 1
MLA, 1 μM	2.9	1.0–8.3	99 ± 4
MLA, 10 μM	4.7	1.2–18.3	101 ± 3
MLA, 100 μM	4.7	1.0–21.8	80 ± 3

n = 6 experiments for nicotine control and 3–5 experiments for all MLA concentrations.

Table 4
Acetylcholine concentration-effect curve EC_{50} and percent maximum response values in TE-671 cells pretreated with 100 μM to 10 pM (d)-tubocurarine (d-TC).

Antagonist pretreatment, molar concentration	ACh[a] EC_{50} (nM)	EC_{50} 95% confidence intervals	Percent maximum response, mean ± S.E.M.
Control, ACh[a]	333.1	134.0–827.1	86 ± 3
d-TC, 10 pM	19.5	0.7–569.2	100 ± 9
d-TC, 100 pM	14.9	2.7–83.5	94 ± 8
d-TC, 1 nM	14.2	1.8–114.4	95 ± 9
d-TC, 10 nM	17.1	2.6–114.0	98 ± 9
d-TC, 100 nM	11.3	1.1–117.6	92 ± 9
d-TC, 1 μM	13.6	1.8–100.9	94 ± 12
d-TC, 10 μM	21.2	3.3–136.4	86 ± 11
d-TC, 100 μM	22.9	0.5–1165.0	45 ± 11

n = 20 experiments for acetylcholine control, and 3–5 experiments for all (d)-tubocurarine concentrations.
[a] Acetylcholine.

3.4. System equilibrium and potentiation of acetylcholine responses

We also investigated the effects of a 30 min MLA preincubation period on the potentiation of 0.1 and 0.01 μM acetylcholine-induced membrane potential sensing dye responses in TE-671 cells. These two concentrations of acetylcholine were chosen because they provided the maximum amount of potentiation relative to control values (Fig. 3A). Each of the four 30 min preincubation experiments contained serial dilutions of MLA from 100 μM to 1.0 pM, a concentration-effect curve for epibatidine added at serial dilutions from 100 μM to 1.0 pM (data not shown), and a control concentration-effect curve for acetylcholine serially diluted from 100 μM to 1.0 pM. As shown in Fig. 5, there was potentiation of both 10 and 100 nM acetylcholine responses in TE-671 cells by all concentrations of MLA tested. Preincubation of TE-671 cells with 0.1 μM MLA increased the membrane potential sensing dye response of 0.1 μM acetylcholine from 18.2 ± 1% of control to a maximum of 81 ± 8% of control. Likewise, the 30 min preincubation of 10 μM MLA prior to the addition of 0.01 μM acetylcholine increased the

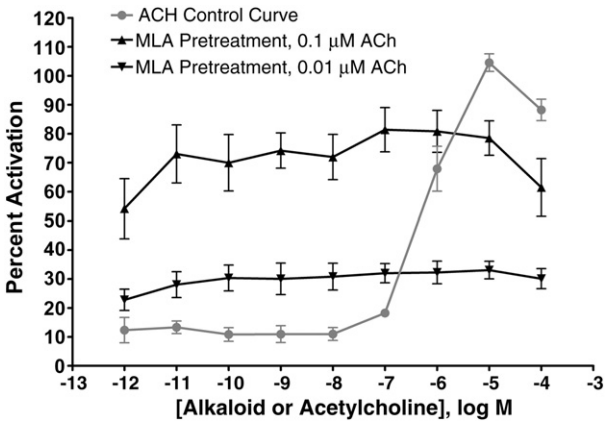

Fig. 5. The effect of 30 min MLA preincubation on the actions of acetylcholine on membrane potential sensing dye fluorescence in TE-671 cells. In each experiment, MLA was serially diluted in 10-fold increments from 100 μM to 1 pM and preincubated at room temperature with TE-671 cells in the 96 well plate for 30 min. Acetylcholine was added at concentrations of either 0.1 or 0.01 μM and the resulting membrane potential sensing dye fluorescence measured and normalized to the maximum epibatidine response. As a control, each of the four experiments contained acetylcholine and epibatidine (data not shown) concentration-effect curves with concentrations of agonist from 0.1 pM to 100 μM. Each datum point represents the mean ± S.E.M. of four independent experiments of duplicate wells.

membrane potential sensing dye response from 11 ± 2% of control to a maximum of 33 ± 1% of control.

3.5. Norditerpenoid alkaloid structure-activity relationships

To control for non-specific effects of MLA on cultured cells or the membrane potential fluorescent dye, we additionally examined the effects of the parent molecule lycoctonine, which lacks the C-18 *N*-methylsuccinimidoanthranoylnilic acid ester moiety of MLA, for its ability to potentiate the response of TE-671 cells to acetylcholine. The membrane depolarization resulting from the addition of acetylcholine in $\log_{10}$ molar concentrations was normalized to the 10 μM epibatidine response. Pretreatment of TE-671 cells with 1 μM lycoctonine did not significantly change the acetylcholine concentration effect relationship (P = 0.248, F test; acetylcholine EC_{50} in presence and absence of 1 μM lycoctonine was respectively 465.1 nM with 95% confidence intervals of 285.2 to 837.8 nM, n = 3, and 399.2 nM with 95% confidence intervals of 165.9 nM to 960.1 nM, n = 20).

3.6. Neostigmine

MLA was tested to determine if it could potentiate the TE-671 cell response to the anticholinesterase agent, neostigmine. The actions of neostigmine on membrane potential in TE-671 cells are shown in Fig. 6A. In the absence of MLA, neostigmine had an EC_{50} of 138.5 μM (95% confidence intervals, 58–328 μM) with a maximum response of 51.8 ± 1.5% at a concentration of 1.0 mM (n = 7). As seen in Fig. 6B, preincubation of TE-671 cells with 100 nM MLA approximately doubled TE-671 cell responses to neostigmine (P<0.0001, ANOVA). However, 100 nM MLA did not significantly increase the TE-671 cell response to 1 mM neostigmine (P>0.05; Tukey's multiple comparison test).

4. Discussion

The present study examined the membrane potential responses of TE-671 cells, which express fetal muscle-type nicotinic cholinergic receptors, to three receptor agonists alone and in combination with MLA. Epibatidine was the most potent agonist and nicotine, the least effective agonist in this study (Tables 1–3). MLA has been shown to act as a partial agonist nicotinic acetylcholine receptors. For example, Cooper et al. (1996) documented that MLA induced single channel currents in *Xenopus* oocytes expressing rat fetal muscle-type nicotinic acetylcholine receptors. However, at concentrations ranging from 0.03 μM to 100 μM, it produced no significant changes in membrane potential (Fig. 1). This result suggests that MLA has no intrinsic efficacy at fetal muscle-type nicotinic acetylcholine receptors expressed by TE-671 cells.

MLA produced sinistral and upward shifts in the concentration-effect relationships of epibatidine, nicotine, and acetylcholine, indicating that it acts to increase the potency and efficacy of these agonists at nicotinic cholinergic receptors. At low MLA concentrations, this action was surmounted by high concentrations of epibatidine and acetylcholine. The surmountability of the actions of MLA by acetylcholine and epibatidine may indicate competitive co-agonistic interactions between MLA and agonists at the receptor ligand binding sites (Smulders et al., 2005; Zwart and Vijverberg, 1997; Zwart and Vijverberg, 2000). The failure of nicotine to surmount MLA action may be associated with the low potency of nicotine at fetal muscle-type receptors. At higher (100 μM) MLA concentrations, cellular responses to acetylcholine and epibatidine were decreased; this phenomenon may be attributable to noncompetitive ion channel block by either MLA or the agonists. Ion channel blockade has been observed with high concentrations of many drugs that act at nicotinic acetylcholine receptors (Lape et al., 2009). In sum, the results of this study suggest that MLA at low concentrations acts as a competitive co-agonist, but is a non-competitive cation channel blocker at higher concentrations. The parent molecule to MLA, lycoctonine, lacks the C-18 *N*-methyl-succinimidoanthranoylic acid ester of MLA and was ineffective in altering cell responses to acetylcholine. This result indicates that the ester moiety of MLA plays an important role in the potentiation of the acetylcholine response in TE-671 cells.

(d)-tubocurarine is a classical antagonist at nicotinic acetylcholine receptors that can activate nicotinic acetylcholine receptors, directly block the ion channel portion of the receptor, or act as a co-agonist to potentiate agonist responses (Ascher et al., 1979; Cachelin and Rust, 1994; Colquhoun et al., 1979; Steinbach and Chen, 1995; Trautmann, 1982; Ziskind and Dennis, 1978). As has been documented by other investigators, we observed that low concentrations of acetylcholine were potentiated by low concentrations of (d)-tubocurarine in a surmountable manner. Likewise, the insurmountable inhibition of

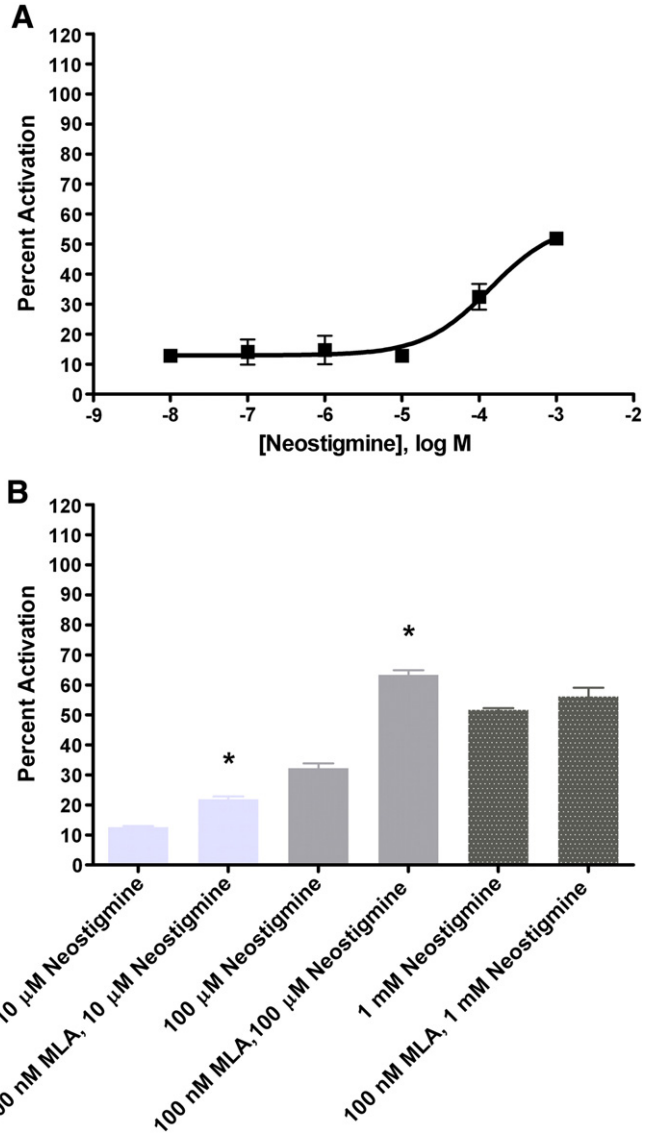

Fig. 6. Concentration-effect relationship for neostigmine (A) and the MLA potentiation (B) of the membrane potential sensing dye response of TE-671 cells to neostigmine. Each datum point represents the mean ± S.E.M. of seven experiments of duplicate wells for the neostigmine concentration-effect curve, or four experiments of duplicate wells for the MLA pretreatment experiments. For comparison, the concentrations of neostigmine which elicited a change in membrane potential sensing dye response in the concentration-effect experiments displayed in panel A serve as the reference responses in panel B. * = P<0.05 MLA pretreated verses control neostigmine concentration (ANOVA, Tukey's multiple comparison test).

acetylcholine responses by 100 μM of (d)-tubocurarine suggests non-competitive antagonism and direct ion channel block of the receptor. It has been proposed that the mechanism underlying (d)-tubocurarine-induced potentiation is the binding of one (d)-tubocurarine molecule and one molecule of agonist to the receptor (Cachelin and Rust, 1994; Steinbach and Chen, 1995). Recent work on the mechanisms underlying co-agonism by Smulders et al. (2005), based on the $\alpha_4\beta_2$ nicotinic acetylcholine receptor, has led to the proposal of an eight-state, two-site receptor occupation model. This model features ligand binding site occupations of agonists and co-agonists, and two ion channel block states induced by agonists and co-agonists. One assumption of this model is that there are two identical ligand-binding sites that must be occupied to activate the receptor. This assumption is contrary to other reports that have documented two nonequivalent (d)-tubocurarine binding sites (Blount and Merlie, 1989). Sine (1988) has defined two ligand binding sites that possessed nearly identical affinities for dimethyl (d)-tubocurarine in TE-671 cells. Based on this latter model, it is possible that there is no subunit/ligand binding site selectivity for (d)-tubocurarine co-agonist activity in our experiments. If the actions of MLA and (d)-tubocurarine are compared (Fig. 3A, B), both possess similar patterns of co-agonist activity, and indeed may have a common mechanism of co-agonist action. The co-agonist actions of MLA with epibatidine were also of a similar pattern to that of acetylcholine suggesting that one molecule of epibatidine and one molecule of MLA can activate the receptor. Caution must be taken when using the model described above with epibatidine because it has been shown to possess higher affinities for the αγ subunit interface ligand binding site than the αδ subunit interface ligand binding site of fetal muscle-type nicotinic acetylcholine receptors and has not been investigated for subunit selectivity in TE-671 cells (Prince and Sine, 1998). Our data do not allow us to speculate about the specific receptor subunits that may play a role in MLA co-agonism.

System equilibrium is an important factor in the measurement of drug activity (Kenakin, 2006). To control for possible equilibrium and temporal effects between MLA and the two ligand binding sites, we increased the incubation time of MLA to 30 min. When 0.1 μM MLA was preincubated for 30 min prior to the addition of 0.1 μM acetylcholine the TE-671 cell membrane potential sensing dye response to the agonist was 81 ± 8% of control. When 0.1 μM MLA was preincubated for 80 s prior to the addition of 0.1 μM acetylcholine the TE-671 cell membrane potential sensing dye response to acetylcholine was 76 ± 6% of control. These responses were not significantly different ($p > 0.05$) and suggest that equilibrium and temporal effects of MLA interactions with the receptor do not play a significant role in the potentiation of acetylcholine responses in TE-671 cells, at least in the two preincubation periods used in this work.

Recent research in our laboratory has shown that the drug neostigmine is effective at reversing the effects of *Delphinium* alkaloids in acutely intoxicated cattle (Green et al., 2009). Therefore, we were interested in determining the actions of MLA on neostigmine in TE-671 cells. Neostigmine is a carbamate acetylcholinesterase inhibitor which has been shown to inhibit the binding of [^{3}H] acetylcholine and ^{125}I-α-bungarotoxin to *Torpedo californica* membranes and act as a partial agonist to stimulate the binding of [^{3}H] perhydrohistrionicotoxin to *Torpedo* channels (Sherby et al., 1985). Functionally, micromolar concentrations of neostigmine have been shown to potentiate the effects of the nicotinic acetylcholine receptor agonist carbachol in PC12 cells (Nagata et al., 1997). These actions are not restricted to neostigmine, other acetylcholinesterase inhibitors such as physostigmine and tacrine can competitively bind nicotinic receptors to potentiate the effects of acetylcholine (Zwart et al., 2000). Neostigmine produced a maximum change in TE-671 cell membrane potential at a concentration of 1.0 mM. Pretreatment of TE-671 cells with 100 nM MLA significantly increased the effect of 10.0 and 100.0 μM neostigmine. Thus, MLA appears to potentiate the actions of neostigmine at $(\alpha_1)_2\beta_1\gamma\delta$ nicotinic acetylcholine receptors in TE-671 cells. The results of other investigators described above coupled with results presented in this report suggest that MLA can act as a co-agonist at the ligand binding sites of fetal muscle-type nicotinic acetylcholine receptors expressed by TE-671 cells.

MLA toxicosis in cattle has been explained by its antagonistic action at nicotinic cholinergic receptors. It has been well documented that cattle grazing in mountain pastures with toxic larkspur repeatedly cycle between one or two days of high consumption of larkspur followed by a period of detoxification (Pfister et al., 1997a). It is tempting to speculate that MLA ingestion in low amounts by cattle may potentiate nicotinic acetylcholine receptors in the central nervous system that are associated with appetitive behavior to facilitate cyclic toxicant consumption. Indeed, brain nicotinic cholinergic receptors have been implicated in feeding behavior (Jo et al., 2002). Conversely, high doses of MLA in cattle may lead to an aversion of larkspur consumption or clinical signs of toxicity and death.

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