Effects of Delphinium Alkaloids on Neuromuscular Transmission

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ABSTRACT

The Delphinium alkaloids methyllycaconitine (MLA), nudicauline, 14-deacetylnudicauline (14-DN), barbinine, and deltaline were investigated for their effects on neuromuscular transmission in lizards. The substituent at C14 provides the only structural difference among the alkaloids MLA, nudicauline, 14-DN, and barbinine. Deltaline lacks the N-(methylsuccinyl)anthranilic acid at C18 common to the other four alkaloids. Each alkaloid reversibly reduced intracellularly recorded compound muscle action potential (CMAP) amplitudes in a concentration-dependent manner. The IC50 values for CMAP blockade were between 0.32 and 13.2 μM for the N-(methylsuccinimido)anthranoyllycaconitine-type alkaloids and varied with the C14 moiety; the IC50 value for deltaline was 156 μM. The slopes of the concentration-response curves for CMAP blockade were similar for each alkaloid except barbinine, whose shallower curve suggested alternative or additional mechanisms of action. Each alkaloid reversibly reduced intracellularly recorded spontaneous, miniature end-plate potential (MEPP) amplitudes. Alkaloid concentrations producing similar reductions in MEPP amplitudes were 0.05 μM for 14-DN, 0.10 μM for MLA, 0.50 μM for barbinine, and 20 μM for deltaline. Only barbinine altered the time constant for MEPP decay, further suggesting additional or alternative effects for this alkaloid. MLA and 14-DN blocked muscle contractions induced by exogenously added acetylcholine. All five alkaloids are likely nicotinic receptor antagonists that reduce synaptic efficacy and block neuromuscular transmission. The substituent at C14 determines the potency and possibly the mechanism of nicotinic acetylcholine receptor blockade for MLA, nudicauline, 14-DN, and barbinine at neuromuscular synapses. The lower potency of deltaline indicates that the N-(methylsuccinyl)anthranilic acid at C18 affects alkaloid interactions with nicotinic acetylcholine receptors at neuromuscular junctions.

Plants belonging to the genus Delphinium have been recognized for their toxic effects on insects and mammals for centuries (Dioscoreides, The Greek Herbal of Dioscorides; Gerard, 1975; Mitton and Mitton, 1982). To this day, cattle are frequently poisoned in the western regions of North America by Delphinium spp. ingestion (Benn and Jacyno, 1983). Methyllycaconitine (MLA), one of the many norditerpenoid alkaloids found in these plants, has been reported to be a competitive antagonist for nicotinic acetylcholine receptors (nAChRs) at mammalian neuromuscular junctions (Dozoretseva, 1959; Nambi-Aiyar et al., 1979). In addition to MLA, North American Delphinium spp. contain numerous toxic alkaloids, including deltaline, nudicauline, 14-deacetylnudicauline (14-DN), and barbinine. The effects of these alkaloids on neuromuscular transmission are largely unknown (Pelleter, 1983; Manners et al., 1993, 1995).

Alkaloids commonly found in Delphinium spp. are derivatives of the norditerpenoid lycoctonine. Among these alkaloids, deltaline is a 7,8-methylenedioxylycoctonine-type (MDL) norditerpenoid alkaloid, whereas MLA, nudicauline, 14-DN, and barbinine are lycoctonine derivatives esterified with N-(methylsuccinyl)anthranilic acid at C18 and designated collectively as N-(methylsuccinimido)anthranoyllycaconitine (MSAL)-type alkaloids (Manners et al., 1993, 1995). The N-(methylsuccinyl)anthranilic acid moiety appears to affect alkaloid toxicity and affinity for nAChR types because norditerpenoid alkaloids lacking this group are at least 100 times less lethal/potent than the MSAL-type alkaloids (Manners et al., 1993, 1995; Hardick et al., 1995, 1996). The only structural difference among these four

ABREVIATIONS: MLA, methyllycaconitine; 14-DN, 14-deacetylnudicauline; nAChR, nicotinic acetylcholine receptor; CMAP, compound muscle action potential; MEPP, miniature end-plate potential; MDL, 7,8-methylenedioxylycoctonine-type; MSAL, N-(methylsuccinimido)anthranoyllycaconitine; EDL, m. extensor digitorum longus; PSS, physiological saline solution; Rm, membrane resistance; τMEPP, miniature end-plate potential time constant; τCMAP, miniature end-plate current time constant.
Materials and Methods

Neuromuscular Preparations and Solutions. Adult lizards (Anolis carolinensis) were deeply anesthetized with halothane and then decapitated. For extracellular recording experiments, the hind limbs were removed, the sciatic nerve was isolated, and all hind limb muscles except the m. extensor digitorum longus (EDL) were removed. The resulting neuromuscular preparation was pinned to a Sylgard-coated 35-mm culture dish and continuously perfused by gravity feed at 2 to 3 ml/min with physiological saline solution (PSS; 122 mM NaCl, 3 mM KCl, 2 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 5 mM HEPES, pH 7.2) at 20–22°C. To record miniature end-plate potentials (MEPPs), intercostal muscles and their attached ribs were removed, pinned to a Sylgard-coated 35-mm culture dish, and perfused with PSS as described above. The free-base form of each MSAL-type alkaloid was solubilized in PSS at pH 4.0 as a 100 μM stock solution. Deltaline was solubilized similarly as a 1 mM stock solution. All stock solutions were frozen at −70°C as 1-ml aliquots. Working alkaloid solutions were made fresh before each experiment by serial dilution and adjustment to pH 7.2.

Extracellular Recording. Compound muscle action potentials (CMAPs) were recorded extracellularly using a bipolar platinum wire recording electrode. Recordings were made by placing the uninsulated tips of the platinum wires on the surface of the EDL muscle and stimulating the sciatic nerve supramaximally with a suction electrode. Recordings were digitized using Super Scope acquisition software (GW Instruments, Somerville, MA), stored on a Macintosh IIci computer, and later analyzed using Microsoft Excel spreadsheet software.

For each experiment, the sciatic nerve-EDL preparation served as its own control. Before alkaloid application, control CMAPs were elicited with 1-Hz stimulation and recorded. A known concentration of alkaloid in PSS was then bath-applied to the preparation for 20 to 30 min before recording CMAPs evoked at 1-Hz stimulation. The alkaloid solution was then washed out by perfusion with normal PSS for 30 to 45 min. After washout, CMAPs were elicited at 1-Hz stimulation and recorded to determine the reversibility of the alkaloid effect. For each experiment, CMAP amplitudes were normalized to the control value obtained for that preparation; two or three concentrations of alkaloid were tested in each preparation.

Intracellular Recording. The effects of 14-DN, MLA, barbinine, and deltaline on MEPPs were studied in lizard (A. carolinensis) intercostal muscle preparations. Each intercostal muscle fiber was used as an internal control. MEPPs were recorded intracellularly using a Warner IE-201 Intracellular Electrometer (Warner Instrument Corporation, Hamden, CT). Signals were filtered at 10 kHz and digitized at 3 kHz using the Super Scope acquisition software. Digitized data were stored on a Macintosh IIci computer and later analyzed using the Microsoft Excel spreadsheet software. MEPPs were gathered in event triggered mode by setting a threshold to twice the amplitude of the background noise, which was typically about 200 μV. Data points acquired from 5 ms before to 20 ms after the threshold setpoint were stored temporarily in a memory buffer, inspected visually, and either accepted and saved to disk or rejected and dumped from the memory buffer. Criteria for acceptance were a rapid rise time (<2 ms) and an apparently exponential decay. This procedure reduced the requisite digital storage space and facilitated analysis of MEPP characteristics but did not permit an analysis of alkaloid effects on MEPP frequency. Manual selection of MEPPs introduced the possibility of bias toward selection of larger-amplitude events. To check this possibility, MEPP amplitude distributions were tested for normalcy. All MEPP amplitudes were normally distributed about the mean, suggesting that our selection method was not biased toward larger-amplitude events.

Microelectrodes were pulled and subsequently filled with a 4 M potassium acetate solution yielding electrodes with resistances of 3 to 6 MΩ. Individual muscle fibers were visualized with either a dissecting or compound microscope and impaled with a microelec-

**Fig. 1.** Norditerpenoid alkaloid structures. Top, skeletal structure of the MSAL-type alkaloids. The R group on C14 provides the only structural difference among the MSAL-type alkaloids tested, and may be O, barbinine; OH, 14-DN; OMe, MLA; or OAc, nudicauline. Bottom, skeletal structure of the MDL-type alkaloid deltalone. Deltalone is hydroxylated at C10 and lacks the N-(methylsuccinimido)anthranilic acid at C-18 that is characteristic of the MSAL-type alkaloids.
trode placed within one muscle fiber diameter of the nerve terminal. The membrane potential was allowed to stabilize for 5 min after impalement, and control recordings were made in muscle fibers with resting membrane potentials between ~75 and ~85 mV. MEPPs were analyzed from muscle fibers that exhibited stable membrane potentials (i.e., less than ±10% variation during the recording session). All preparations were continuously perfused with PSS. After control recordings were made and stored, each preparation was perfused with alkaloid-containing PSS for 30 min before taking recordings to measure the effects of alkaloid on MEPP amplitude. After alkaloid administration, the preparation was perfused in normal PSS for 30 min before recordings were taken to measure recovery.

For each muscle fiber, between 50 and 100 MEPPs were recorded for each of the following conditions: before alkaloid application, in the presence of alkaloid, and after the 30-min wash whenever possible. For each alkaloid, a concentration was used that reduced MEPP amplitude about 30 to 40%. Reducing MEPP amplitude by more than this amount often yielded potentials too small to be distinguished from background noise.

Acetylcholine-Induced Muscle Contraction. To investigate the ability of MLA, 14-DN, and delteline to prevent acetylcholine-induced muscle contraction, intercostal muscles were pinned out as described above in a bath volume of about 250 μl. Acetylcholine (100 μM) was manually applied directly above the muscle preparation. The volume of acetylcholine was adjusted to obtain reliable muscle contraction and was typically 5 μl. The interval between acetylcholine applications was 5 min to avoid receptor desensitization. After reliable muscle contraction was achieved, 14-DN (5 μM), MLA (10 μM), or delteline (500 μM) was perfused into the bath. Alkaloid concentrations were chosen to ensure complete blockade of neuromuscular transmission. After a 20- to 45-min incubation in alkaloid, acetylcholine accompanied by alkaloid was again applied, and muscle contraction was monitored visually through a dissecting microscope. To ensure that none of the alkaloids affected direct stimulation of muscle contraction, 25 μl of osmotically adjusted PSS containing 50 mM K+ was manually added to the bath in the presence of alkaloid after alkaloid blockade was achieved. Alkaloids were removed by bath perfusion with normal PSS for 30 min, and acetylcholine-induced contractions were again monitored to ensure preparational viability and reversibility of the alkaloid effect.

Data Analysis. Measurements of CMAP amplitudes were taken as peak-to-peak values. Concentration-dependent inhibition curves were fit to the CMAP data using the equation

$$R = \frac{R_{\text{max}}}{1 + \left(\frac{[\text{alkaloid}]}{IC_{50}}\right)^n}$$

where $R$ is the fractional response, $R_{\text{max}}$ is the response recorded in the absence of alkaloid, [alkaloid] is the alkaloid concentration, $n_{50}$ is the slope of the curve, and $IC_{50}$ is the alkaloid concentration that produces a 50% inhibition of the maximal response. Curves were iteratively fit by allowing the $IC_{50}$ value and slope of the curve to float. Slopes and $IC_{50}$ values were obtained from the best-fit curves using the least-squares method.

The mean values for MEPP amplitudes were determined for each muscle before during and after exposure to alkaloid and compared using one-way ANOVA. To assess variability in MEPP amplitude, the coefficient of variation (S.D./mean; Martin, 1966) was calculated for each muscle fiber before alkaloid addition, in the presence of alkaloid, and after alkaloid removal. The values for the coefficient of variation were compared using a Student’s t-test. To measure MEPP decay constants, groups of 11 to 15 MEPPs recorded in the same muscle fiber and under the same conditions were signal averaged by aligning the rising phases of the digitized, event-triggered MEPPs in an Excel spreadsheet and averaging the corresponding data points of each MEPP in the group. The decay constant was determined by fitting the decay phase of the MEPPs to an exponential function. Decay constants under control and experimental conditions were compared using ANOVA to determine whether the alkaloids affected the MEPP decay constant.

Results

The use of extracellular and intracellular recording techniques in lizard muscles permitted an analysis of alkaloid effects on action potential generation and nAChR function at neuromuscular synapses. Because the lizard EDL is a small, nearly cylindrical muscle that yields a high current density, extracellular wire electrodes could be used to investigate alkaloid effects on action potential generation in a population of muscle fibers. Lizard intercostal muscles were used to investigate alkaloid effects on nAChRs. This preparation is especially well suited for intracellular recording and pharmacological studies because end-plate regions are easily visualized and diffusion barriers are minimized in this one-muscle-fiber-layer-thick muscle. However, the arrangement of muscle fibers in a thin sheet precluded the use of extracellular recording techniques to measure the nearly simultaneous generation of action potentials in populations of muscle fibers. Alkaloid effects on nAChR function are comparable among skeletal muscles because postsynaptic nAChRs are similar regardless of the muscle type (Salpeter, 1987). Cross-species comparisons of alkaloid effects on neuromuscular transmission are also practical because nAChR function at the neuromuscular junction is similar regardless of the vertebrate species (Salpeter, 1987).

Alkaloid Blockade of CMAPs. Compound muscle action potentials were elicited in an isolated lizard EDL through sciatic nerve stimulation (Fig. 2). For each experiment, the stimulus strength was increased until the CMAP peak-to-peak amplitude reached a maximum, indicating that the number of muscle fibers reaching threshold had been maximized. Reducions in maximal CMAP amplitude after exposure to alkaloid indicated a decrease in the number of muscle fibers brought to threshold by nerve stimulation and provided a measure of the ability and potency of each alkaloid to block neuromuscular transmission (Fig. 2).

All of the alkaloids produced a concentration-dependent reduction in the amplitude of the nerve-evoked CMAP (Fig. 3). Alkaloid-induced changes in CMAP amplitudes were expressed as a percent of the control CMAP amplitude obtained before alkaloid application. For each alkaloid, the reduction in CMAP amplitude was fully reversible by washing in normal PSS for 30 min (Fig. 2).

To establish concentration-response curves, CMAP amplitudes were measured at two or three alkaloid concentrations in four or five neuromuscular preparations for each of the five alkaloids. In cases where multiple data points were obtained for the same alkaloid concentration, the normalized CMAP amplitudes were averaged (Fig. 3). The $IC_{50}$ values obtained from these measurements ranged from 0.32 μM for nudicauline to 156 μM for delteline (Table 1). All four MSAL-type alkaloids were more potent than delteline (Table 1). The order of alkaloid potency (most to least) for the reduction of CMAP amplitude was nudicauline, 14-DN, MLA, barbinine, and delteline (Fig. 3 and Table 1). Nudicauline, 14-DN, MLA, and delteline displayed similarly steep concentration-depen-
inhibition curves for blockade of nerve-evoked CMAPs and exhibited slopes between 2.53 and 3.62. In contrast, the slope for barbinine was close to 1.5 (Table 1).

**Alkaloid Blockade of MEPPs.** Alkaloid-induced changes in MEPP amplitude were determined for 14-DN, MLA, barbinine, and deltaline (Fig. 4 and Table 1). Nudicauline was not studied because insufficient quantities of the alkaloid were available at the time. Measurements of MEPP amplitudes provided a more direct determination of alkaloid effects on nicotinic receptors than measurements of CMAP amplitude because a MEPP occurs when the contents of a single synaptic vesicle opens nicotinic receptor channels in the postsynaptic membrane (Anderson and Stevens, 1973; Kuffler and Yoshikami, 1975; Salpeter, 1987). All of the alkaloids tested significantly reduced (p < .05 to p < .001) the mean MEPP amplitude compared with the control state (Tables 1 and 2). The relative potency for this reduction was the same as that for the reduction of CMAP amplitudes (Table 1 and Fig. 4). After alkaloid washout, the mean MEPP amplitude was not different compared with controls for any of the alkaloids (Table 2). For 14-DN, barbinine, and deltaline, the alkaloid effect appeared to be completely reversible because the mean MEPP amplitude after alkaloid washout was greater than that in the presence of alkaloid (p < .05) and not different from controls (p > .19). However, for MLA, the alkaloid effect appeared to be incompletely reversible because mean MEPP amplitudes after washout, although larger than in the presence of alkaloid, were not significantly different from one another (p = .09). When the amplitudes of all of the MEPPs collected in the MLA studies were compared rather than comparing the mean values for each treatment, the amplitudes were significantly larger before and after alkaloid administration.

Incomplete reversal of the alkaloid effect after washout could result from a time-dependent degradation of the neuromuscular preparation during the course of the experiment. To test this possibility, muscle fiber impalements were maintained for up to 3 h, and MEPPs were recorded at 15- and 30-min intervals during this period. MEPP amplitudes remained constant for up to 2 h after microelectrode impalement (data not shown). These results indicate that the incomplete reversal was due to an incomplete washout of the alkaloid and not irreversible blockade of the receptors or time-dependent degradation of the muscle fiber preparation. Ordinarily, the population of MEPP amplitudes is distributed normally about the mean amplitude (Boyd and Martin, 1956b). If the alkaloids impair synaptic transmission by blocking postsynaptic receptor function, their effect on MEPP amplitude would be to reduce the mean amplitude without affecting the distribution of amplitudes about the new, lower mean. Conversely, the alkaloids could impair synaptic transmission by affecting synaptic vesicle loading and decreasing quantal size. To distinguish between presynaptic and postsynaptic effects of Delphinium alkaloids, cumulative frequency histograms were plotted for MEPP amplitudes (Fig. 5, a–d). For each alkaloid, the mean MEPP amplitude was reduced from controls (Table 2), and none of the alkaloids affected the distribution of MEPP amplitudes around the mean (Fig. 5, a–d). The coefficient of variation for MEPP amplitudes ranged between 0.13 and 0.23. The coefficient of variation was the same in the presence and absence of alkaloid (Student’s t test, p > .15), further suggesting that the alkaloids had no effect on the quantal size (Table 2). These results suggest that the alkaloids impair neuromuscular transmission by blocking the nAChRs on the postsynaptic membrane and that they do not affect the loading of neurotransmitters into synaptic vesicles.
but significant (P, illustrated responses were 0.1 which is an average of 11. The alkaloid concentrations that produced the trace is an average of 15 MEPPs, except for the deltaline-treated trace, increased (Table 3). Esterase blockade increases intercostal muscle contraction. Incubation in MLA (10 M) for up to 45 min failed to prevent muscle contraction induced by this method. Muscle contraction produced by elevated extracellular K+ was unaffected by incubation in any of the three alkaloids.

The low signal-to-noise ratio for intracellularly recorded MEPPs precluded a direct determination of concentration-response curves to quantify the relationship between alkaloid concentration and MEPP amplitude reduction. As an alternative, alkaloid-induced reductions in MEPP and CMAP amplitudes were correlated. Alkaloid concentrations that produced proportional reductions of MEPP and CMAP amplitudes were compared for each alkaloid (Fig. 6). For example, 0.1 M MLA, which reduced MEPP amplitudes by 29%, was compared with the concentration of MLA that reduced the CMAP amplitude by 29% (Figs. 2 and 6). Linear regression analysis of these points yielded a statistically significant correlation (r = 0.99) between alkaloid concentrations, producing proportional reductions in MEPP and CMAP amplitudes (Fig. 6). This result suggests that for each alkaloid, the blockade of neuromuscular transmission results from alkaloid inhibition of nAChR function at the neuromuscular synapse. Because of the safety factor for neuromuscular transmission, equivalent blockade of CMAP and MEPP amplitudes required about 11 times more alkaloid for CMAPs than for MEPPs (Figs. 2 and 1).

The alkaloids were also examined for possible agonist/partial agonist properties. Bath application of the alkaloids at the concentrations used in this study did not cause muscle contraction or changes in the muscle fiber resting membrane potentials. In contrast, bath application of 0.1 M (l)-nicotine caused a visually recognizable muscle contraction (data not shown). Based on these findings, alkaloid concentrations sufficient to block neuromuscular transmission did not cause ion channel openings sufficient to depolarize muscle fibers.

*Delphinium* alkaloids block nAChRs in the central and peripheral nervous systems (Ward et al., 1990; Sargent, 1993; Albuquerque et al., 1997; Dobelis et al., 1997), but the principal signs of *Delphinium* toxicity (paresis and paralysis) are consistent with an alkaloid-induced blockade of neuromuscular transmission. As a simple test of this possibility, the alkaloid IC50 values obtained in the current study were correlated with previously published LD50 values from mammals (Manners et al., 1995). This comparison yielded a correlation coefficient of 0.98 (Fig. 7), suggesting that each alkaloid exerts its toxic effects through a similar mechanism. Although this correlation suggests that alkaloid lethality could result from nAChR blockade at the neuromuscular junction, additional studies will be required to rule out alternative sites for *Delphinium*-induced lethality and differences in species susceptibility to *Delphinium* poisoning.
Electrophysiological measurements were used to characterize the effects of the Delphinium norditerpenoid alkaloids MLA, nudicauline, 14-DN, barbinine, and deltaline on synaptic transmission at lizard neuromuscular junctions. Neuromuscular synapses were selected for these studies because a single type of nAChR mediates neuromuscular transmission and because the clinical signs of Delphinium poisoning are consistent with a curariform block of this receptor. In addition, MLA is known to interact with muscle-type nAChRs (Dozortseva, 1959; Nambi-Aiyar et al., 1979; Ward et al., 1990; Garcha et al., 1993; Yum et al., 1996; Tian et al., 1997). The IC50 value obtained for MLA-induced CMAP blockade in lizards (1.5 μM) is in good agreement with the previously reported IC50 value (2.3 μM) for blockade of nerve-evoked twitch in rat diaphragm (Nambi-Aiyar et al., 1979). All five alkaloids blocked neuromuscular transmission in a concentration-dependent manner, and the relative potencies among the alkaloids were similar to those reported for in vivo toxicities in mammals (Manners et al., 1995).

Nudicauline, 14-DN, MLA, and deltaline reduced CMAP amplitudes with similarly steep concentration-dependent relationships (Table 1). The similarity in these slopes and the similar characteristics for MEPP amplitude reduction suggest that these four alkaloids block neuromuscular transmission via the same mechanism. The finding that MLA and 14-DN block acetylcholine-induced muscle contraction indicates that these alkaloids block nAChRs at the neuromuscular junction. The results of our studies combined with the well-established competitive interaction of MLA with muscle-type receptors (Dozortseva, 1959; Nambi-Aiyar et al., 1979; Ward et al., 1990; Garcha et al., 1993; Yum et al., 1996; Tian et al., 1997) suggest that nudicauline, 14-DN, and deltaline are likely competitive nAChR antagonists at the neuromuscular junction.

All of the Delphinium alkaloids reduced the mean MEPP amplitude without affecting the distribution of amplitudes about the mean, suggesting that these alkaloids act postsynaptically to reduce synaptic efficacy. The amplitude of the postsynaptic potential is proportional to the number of open channels when its amplitude is less than 10% of the resting membrane potential (McLachlan and Martin, 1981). MEPP amplitudes were usually between 0.5 and 0.75 mV, whereas resting membrane potentials were between −75 and −85 mV. Alkaloid concentrations insufficient to block neuromuscular transmission decreased MEPP amplitudes by about 30 to 40%, suggesting that the alkaloids reduced the number of open nAChRs by a similar amount. The leftward shift of the curves in the cumulative frequency histograms (Fig. 5, a–d) is consistent with this interpretation.

The values for the coefficient of variation in MEPP amplitudes reported here are similar to those reported for mammalian neuromuscular junctions (Boyd and Martin, 1956a). Alkaloid-induced changes in the coefficient of variation would be consistent with variability in synaptic vesicle load-
A linear regression analysis yielded a correlation coefficient of 0.98. This interpretation is consistent with recent findings showing that MLA reduces spontaneous miniature end-plate currents without altering the time course of their decay in rat diaphragm (Tian et al., 1997). The similarity in the effects of MLA, 14-DN, and deltaline on MEPP amplitude, MEPP amplitude distribution, and MEPP decay constant is consistent with these alkaloids competing with acetylcholine for a common binding site on the nAChRs at the neuromuscular junction.

The high correlation coefficient obtained by comparing alkaloid concentrations equipotent for reducing CMAP and MEPP amplitudes supports the hypothesis that the MSAL and MDL alkaloids impair neuromuscular transmission by blocking postsynaptic nAChRs. An MEPP results when a quantum of neurotransmitter opens closely packed postsynaptic nAChRs. A block of the receptor by nAChR blockade; however, this assay may be inadequate to measure the effects of deltaline on exogenous acetylcholine-induced muscle contraction because of the low potency of deltaline for blocking CMAPs and reducing MEPP amplitudes.

As a more direct test of the ability of alkaloids to block nAChRs, acetylcholine-induced muscle contraction was assayed in the presence of MLA, 14-DN, and deltaline. Both MLA (10 μM) and 14-DN (5 μM) blocked muscle contractions induced by exogenously applied acetylcholine but had no effect on contractions induced by high K+ PSS. This result indicates that these two alkaloids block muscle contraction by blocking nAChRs. Deltaline (500 μM) failed to block exogenous acetylcholine-induced muscle contraction. This result appears to be inconsistent with deltaline-induced nAChR blockade; however, this assay may be inadequate to measure the effects of deltaline on exogenous acetylcholine-induced muscle contraction because of the low potency of deltaline for blocking CMAPs and reducing MEPP amplitudes.

The highly significant correlation between the LD₅₀ and IC₅₀ values for CMAP blockade. The IC₅₀ values obtained in the present study (Table 1) were plotted against previously published LD₅₀ values in mammals (Manners et al., 1995). Linear regression analysis yielded a correlation coefficient of 0.99. This correlation suggests that the alkaloids exert similar effects on the neuromuscular junction, implying a presynaptic effect. None of the alkaloids affected the coefficient of variation (Table 2), indicating that quantal size remained uniform in the presence of alkaloids. These results are consistent with postsynaptic nAChR blockade.

The alkaloids 14-DN, MLA, and deltaline reduced MEPP amplitudes without affecting the resting membrane potential, the rate of MEPP decay, or the coefficient of variation of MEPP amplitude. These results suggest that all three alkaloids act by blocking nAChRs and not by altering either muscle fiber passive membrane properties or neurotransmitter release. This interpretation is consistent with recent findings showing that MLA reduces spontaneous miniature end-plate currents without altering the time course of their decay in rat diaphragm (Tian et al., 1997). The similarity in the effects of MLA, 14-DN, and deltaline on MEPP amplitude, MEPP amplitude distribution, and MEPP decay constant is consistent with these alkaloids competing with acetylcholine for a common binding site on the nAChRs at the neuromuscular junction.

The high correlation coefficient obtained by comparing alkaloid concentrations equipotent for reducing CMAP and MEPP amplitudes supports the hypothesis that the MSAL and MDL alkaloids impair neuromuscular transmission by blocking postsynaptic nAChRs. An MEPP results when a quantum of neurotransmitter opens closely packed postsynaptic nAChRs (Stiles et al., 1996), and CMAP blockade at a site or sites other than nAChRs would yield little or no correlation between receptor and action potential blockade. For example, blockade of voltage-gated Ca²⁺ channels that govern transmitter release would block evoked transmitter release but be independent of effects on the amplitude of spontaneous MEPPs. Similarly, blockade of voltage-gated Na⁺ channels would block neuromuscular transmission by preventing action potential propagation without affecting MEPP amplitude.

As a more direct test of the ability of alkaloids to block nAChRs, acetylcholine-induced muscle contraction was assayed in the presence of MLA, 14-DN, and deltaline. Both MLA (10 μM) and 14-DN (5 μM) blocked muscle contractions induced by exogenously applied acetylcholine but had no effect on contractions induced by high K⁺ PSS. This result indicates that these two alkaloids block muscle contraction by blocking nAChRs. Deltaline (500 μM) failed to block exogenous acetylcholine-induced muscle contraction. This result appears to be inconsistent with deltaline-induced nAChR blockade; however, this assay may be inadequate to measure the effects of deltaline on exogenous acetylcholine-induced muscle contraction because of the low potency of deltaline for blocking CMAPs and reducing MEPP amplitudes.

The highly significant correlation between the LD₅₀ and IC₅₀ for neuromuscular blockade is consistent with the hypothesis that Delphinium alkaloids exert their lethal effects by blocking nAChRs at neuromuscular junctions on skeletal muscle fibers. However, synaptic transmission in autonomic ganglia requires functional nAChRs. MLA exhibits nanomolar affinity for α7 nAChRs found in autonomic ganglia, but blockade of the α7 nAChRs alone fails to block
Functional Implications of Alkaloid Structure. Measurements of alkaloid effects on CMAP and MEPP amplitude provided a functional assay for investigating the relationships between alkaloid structure and alkaloid function. Earlier binding studies on nAChRs from the central nervous system related the chemical substitution at C14 of the alkaloid to alkaloid affinity for these nicotinic receptors (Kukel and Jennings, 1994; Hardick et al., 1996). The rank order of potency for blockade of CMAPs and MEPPs at the neuromuscular junction is consistent with binding studies on neuronal nAChRs (Hardick et al., 1995, 1996, Dobelis et al., 1997). However, the relationship between the moiety substituted at C14 and alkaloid potency in the functional assays is not immediately clear (Fig. 1 and Table 1). If differences in alkaloid potency result simply from steric interactions of the C14 substitutions, the predicted order of potency would be either nudicauline > MLA > 14-DN > barbinine, or the reverse. The order of potency for diminishing CMAP amplitudes was nudicauline > 14-DN > MLA > barbinine. Except for nudicauline, which was not tested against MEPPs, this rank order of potency was the same for reducing MEPP amplitudes.

Barbinine, which was the least potent of the MSAL-type alkaloids, differs structurally from the other MSAL-type alkaloids, having a ketone group at C14 rather than a hydroxyl or an ether group. This substitution changes the hybridization state of C14 from sp³, which imparts a relatively flexible tetrahedral configuration, to a more rigid sp² state. The decreased flexibility could affect the spatial relationship of the tertiary nitrogen and anthranilic-ester groups that are believed to interact with the nAChR ligand-binding site (Ward et al., 1990). This structural change could account for barbinine’s lower potency and possibly different mechanism of action.

The finding that deltalone, which lacks the N-(methylsuc- cinyI)anthranilic acid on C18, is far less potent for CMAP and MEPP blockade than any of the MSAL-type alkaloids is consistent with earlier (¹²⁵I)-α-bungarotoxin competition binding studies that demonstrated a role for the anthranil ester moiety in alkaloid-receptor interactions for neuronal nAChRs (Kukel and Jennings, 1994; Hardick et al., 1995, 1996; Dobelis et al., 1997). The results of the study presented here indicate that MSAL-type alkaloids can be used to distinguish experimentally α1- and α7-containing nAChRs in either in vivo or in vitro preparations that contain a mixture of these receptors. However, such studies require the judicious selection of the appropriate alkaloid concentrations.

The differences in alkaloid structure that produced differences in alkaloid potency for blocking the nAChR type at the neuromuscular junction may prove useful for dissecting the functional significance of the myriad nAChRs in the mammalian central nervous system.


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