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The effects of an adenylate cyclase inhibitor on the electrophysiological correlates of neuromuscular transmission in the frog

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1 The presynaptic and postsynaptic effects of MDL 12,330A, an adenylate cyclase inhibitor in several biological tissues, were studied at motor endplates in frog cutaneous pectoris nerve-muscle preparations.

2 This agent increased both spontaneous quantal acetylcholine (ACh) release and neurally-evoked ACh release approximately twofold during the first 20–40 min of application.

3 The increased ACh release was accompanied by a profound irreversible depression in the amplitudes of the miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps).

4 The response to iontophoretically-applied ACh was reduced in parallel with the amplitude of the spontaneous m.e.p.ps, indicating that the depression of synaptic transmission was postsynaptic in origin.

5 Endplates were voltage-clamped to study the postsynaptic depression in more detail. It was observed that the peak endplate current (e.p.c.) was depressed without concomitant changes in: (a) the kinetics of e.p.c. decay, (b) the relationship between peak e.p.c. and membrane potential, (c) the ACh equilibrium potential or (d) the voltage sensitivity of the e.p.c. decay. This suggests that MDL 12,330A reduces the postsynaptic sensitivity to ACh by a voltage-independent block of the cholinoceptor.

6 The presynaptic enhancement and the postsynaptic depression of junctional transmission produced by MDL 12,330A are discussed in conjunction with current theories of the role of adenylate cyclase and cyclic nucleotides at nicotinic cholinergic synapses.

Introduction

MDL 12,330A, formerly named RMI 12,330A, is a cyclo-alkyl lactamide derivative that has been shown to inhibit adenylate cyclase in a variety of vertebrate tissues (Guellaen *et al.*, 1977; Grupp *et al.*, 1980; Hunt & Evans, 1980). At the motor nerve ending, this agent has been found to occlude the inhibitory effects of adenosine receptor agonists but not the effects of inorganic Ca channel blockers on acetylcholine (ACh) release (Silinsky, 1984). In view of the suggestions that adenylate cyclase and adenosine 3':5'-cyclic monophosphate (cyclic AMP) are involved in the regulation of nicotinic cholinergic synapses (Goldberg & Singer, 1969; Standaert & Dretchen, 1979; Huganir & Greengard, 1983), we decided to study the presynaptic and postsynaptic effects of MDL 12,330A at the frog neuromuscular junction. The results of such an investigation demonstrate that prejunctionally this agent increases both spontaneous and evoked ACh release whilst postjunctionally it causes a profound, irreversible block of the effects of ACh without any concomitant changes in the selectivity of the ACh-operated ion channel or the kinetics of the ACh-receptor interaction.

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Methods

Electrophysiological aspects

Cutaneous pectoris nerve-muscle preparations of frog (*Rana pipiens* or *Rana temporaria*) were dissected and superfused with Ringer solution. Supramaximal stimulation pulses were delivered to the nerve supply through a suction electrode. Intracellular recordings

were made at endplate regions using glass microelectrodes filled with 3 M KCl and with resistances from 5–15 MΩ. Signals from the microelectrodes were fed into a conventional high input impedance preamplifier, the output of which was delivered into an oscilloscope, a pen recorder (Brush-Gould), and an FM tape recorder. Periodically, endplate potential (e.p.p.) amplitudes were averaged by a computer of average transients (Biomac) and the average displayed on the pen recorder (e.g., Figure 1). Miniature endplate potential (m.e.p.p.) frequencies were determined from pen records and m.e.p.p. amplitudes from pen records after first being recorded on magnetic tape. ACh was applied to the endplate region by brief iontophoretic pulses from a microelectrode filled with 1 M ACh (Nastuk, 1953).

Voltage clamp

The two microelectrode voltage clamp method (Takeuchi & Takeuchi, 1959) was employed for recording endplate currents (e.p.cs). Current and voltage microelectrodes were filled with 3 M KCl and had resistances of approximately 5 MΩ. Before the voltage clamp studies, the preparation was first bathed in normal Ringer containing 2 M formamide for 40–50 min and then rewashed with formamide-free solution for 1 h; this procedure eliminates muscle contraction by impairing excitation-contraction coupling (del Castillo & de Motta, 1978). For details of the voltage clamp protocol, see Figure legends. E.p.c. amplitudes and time constants were determined from digitized e.p.cs on-line using a PDP 11 computer. Time constants for e.p.c. decay were obtained by computer-fitting of the falling phase of the e.p.c. using the method of least squares. Standard methods were used to determine statistical significance (see Silinsky, 1984).

Composition of solutions and chemicals

Normal frog Ringer, which was used for the iontophoretic studies, contained (mM): NaCl 115, KCl 2, $CaCl_2$ 1.8, $NaHCO_3$ 2 (pH 7.2–7.4). For the presynaptic study, low Ca/high Mg Ringer contained 0.35–0.4 mM $CaCl_2$, 3–6 mM $MgCl_2$, and 1 mg l^{-1} neostigmine methylsulphate to increase the size of the m.e.p.ps. In some of the voltage clamp studies, the normal Ringer contained (mM): NaCl 111, KCl 4.7, $CaCl_2$ 2, HEPES (N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid). The pH was adjusted to 7.35 by using appropriate amounts of the free acid and Na salt of HEPES.

Formamide, neostigmine methylsulphate and acetylcholine chloride were purchased from the Sigma Chemical Co. (St. Louis MO, U.S.A.). MDL 12,330A ([N-(*cis*-2-phenylcyclopentyl) azacyclotridecan-2-imine hydrochloride) was received as a generous gift from Dr C.R. House in Edinburgh, who obtained it from Merrill Drugs Limited, Cincinnati, Ohio.

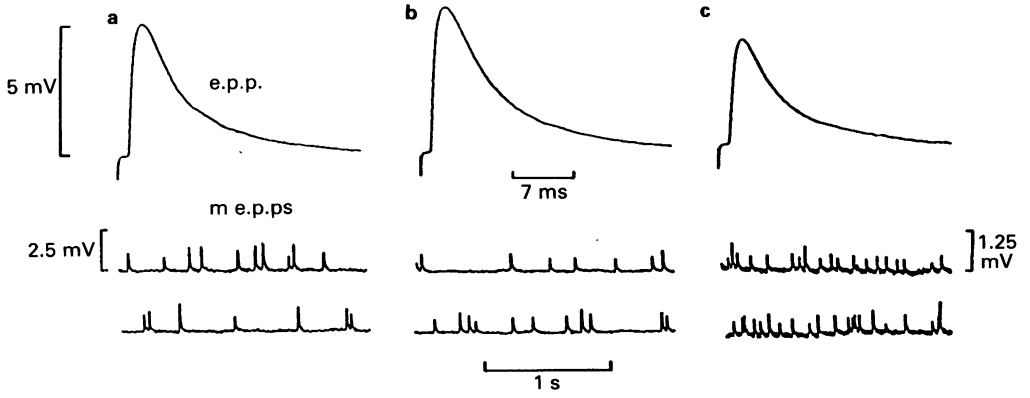

Figure 1 The effect of MDL 12,320A on acetylcholine (ACh) release. Lower traces show miniature endplate potentials (m.e.p.ps), upper traces show computer-averaged endplate potentials (e.p.ps) in response to 32 stimuli at 0.4 Hz. Ringer solution contained 0.35 mM Ca and 6 mM Mg. (a) Control m.e.p.p. frequency = 4.3 ± 0.3 s^{-1} (mean ± s.e.mean, n = 36 for m.e.p.ps in a–c); mean number of quanta released synchronously (M) for the averaged e.p.p. = 4. (b) 10 min after beginning superfusion with 20 μM MDL 12,330A-m.e.p.p frequency = 4.3 ± 0.3 s^{-1}; e.p.p. reflects M = 6. (c) 20 min in 20 μM MDL 12,330A, m.e.p.p. frequency = 7.7 ± 0.4 s^{-1}; M of e.p.p. = 9.8. For m.e.p.p amplitudes, left vertical calibration refers to (a) and (b), right vertical calibration refers only to (c). Note the decline in m.e.p.p. amplitudes in the presence of drug (see text).

Results

General observations: increases in ACh release and a decline in junctional potential amplitudes in the presence of MDL 12,330A

Figure 1 illustrates the typical effect of MDL 12,330A on a preparation in which endplate potentials (e.p.ps) were reduced below threshold using low Ca/high Mg solutions. In this experiment, the m.e.p.p. frequency increased from $4.3\,s^{-1}$ in the control condition (Figure 1a) to $7.7\,s^{-1}$ after 20 min in the presence of 20 μM MDL 12,330A (Figure 1c). A continual decline in m.e.p.p. amplitude was noted during the period of exposure; the m.e.p.p. amplitude fell from the control value of 1.3 mV (a) to 1.0 mV (b) and 0.4 mV (c) and then subsequently to immeasurably small values. With respect to neurally-evoked release, the mean number of ACh quanta released synchronously (M) was determined from the ratio of the e.p.p. to the m.e.p.p. amplitude and this ratio continued to increase during the period at which measurements were possible. Specifically, in the experiment of Figure 1, M increased from the control level of $M = 4$ (Figure 1a) to $M = 6$ (b) after 10 min of superfusion with drug. After 20 min in MDL 12,330-containing solution, M increased further to 9.8 (c). In all seven experiments, during the first 20–40 min in 2–57 μM MDL 12,330A, increases in M ranged from 1.3–2.9 times the control and m.e.p.p. frequency from 1.4–3.3 times control with the mean M and m.e.p.p. frequencies approximately doubling during the period of study.

When exposure to MDL 12,330A, was continued, e.p.ps were also eliminated, even when the preparation was bathed in normal (1.8 mM) or elevated (5.4 mM) Ca without any blocking agent. This effect was largely irreversible and was never associated with any changes in the resting membrane potential of the muscle cell or the holding current under voltage clamp conditions; it will be shown below that this loss of junctional electrical activity is a postsynaptic effect.

The inhibitory effects of MDL 12,330A on potentials produced by the iontophoresis of ACh

In an attempt to determine if the persistent decline in e.p.p. and m.e.p.p amplitudes was postsynaptic in origin, we studied the effect of MDL 12,330A on the response to iontophoretic application of ACh (the iontophoretic potential). Figure 2a shows that, in the absence of an anticholinesterase, superfusion with 5 μM MDL 12,330A reduces the iontophoretic potential in a time-dependent manner. In another experiment (Figure 2b), the Ringer solution contained neostigmine and the decay of both the iontophoretic potential and m.e.p.ps were followed in the same fibre; note the parallel fall in the amplitude of the iontophoretic potential and the m.e.p.p. during superfusion with 7 μM MDL 12,330A. These results, and similar results observed in 4 other experiments demonstrate that the decline of the e.p.ps and m.e.p.ps is due to a

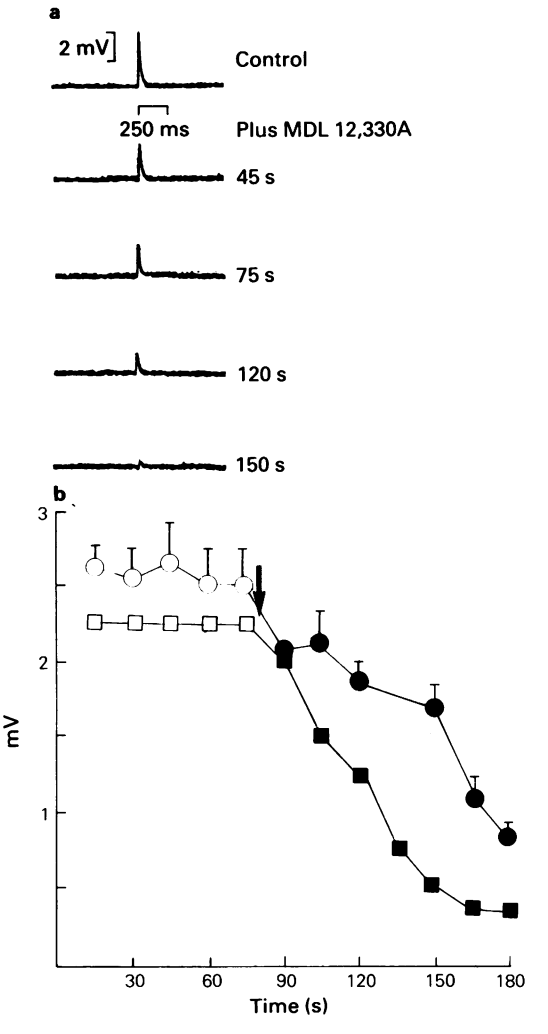

Figure 2 Effects of MDL 12,330A on potentials produced by the iontophoretic application of acetylcholine (ACh). (a) Shows decline of the iontophoretic potential at indicated times after superfusion with 5 μM MDL 12,330A. (b) Shows a parallel decline in the iontophoretic potential (circles) and m.e.p.p. amplitudes (squares) during exposure to 7 μM MDL 12,330A in another experiment. Filled symbols indicate presence of drug (MDL 12,330A applied at the arrow). Experiments shown in both (a) and (b) were made in normal Ringer, neostigmine was present only in (b). In (b) each symbol represents the mean m.e.p.p. amplitude of 11–23 m.e.p.ps and vertical lines indicate s.e.mean.

reduction in the postsynaptic effects of released ACh in the presence of MDL 12,330A. Such postsynaptic blocking effects were observed within 15 min of the beginning of superfusion with concentrations of MDL 12,330A in the hundred nanomolar range but were generally studied at higher concentrations of drug. The decline in postsynaptic sensitivity was irreversible; after treatment with 7–70 μM MDL 12,330A for 2–30 min, no recovery of e.p.ps was observed in a total of 5 experiments, even after 8 h in normal Ringer solution free of drug.

Voltage clamp studies on endplate currents

Possible causes of the reduction in e.p.ps, m.e.p.ps and iontophoretic potentials include a change in ion-selectivity of the endplate membrane, a voltage-dependent block of the ACh receptor or ion channel and a voltage-independent block of the ACh receptor-ion channel complex. To attempt to distinguish between these possibilities, we voltage-clamped the endplate and studied the underlying endplate currents (e.p.cs).

Figure 3 illustrates a family of e.p.cs recorded before (a) and after (b) 3 min of treatment with 10 μM MDL 12,330A. Each e.p.c. was evoked by stimulating the nerve supply 50 ms after the membrane potential was stepped to a given potential (V_m, see Figure legend). Note the reduction in e.p.c. peak at all levels of V_m by MDL 12,330A. The experiment of Figure 3 is illustrated more fully in Figure 4, which shows a plot of the peak e.p.c. against V_m.

The results shown in Figures 3 and 4 demonstrate that the e.p.c. is depressed at each V_m without a change in reversal potential (arrow in Figure 4) and suggest that the inhibitory effect of MDL 12,330A is unlikely to be produced by a change in the cation selectivity of the ACh operated ion channel. Such changes in selectivity would be accompanied by a change in the reversal potential of the e.p.c. in the presence of MDL 12,330A and this was not observed in any of the five experiments. The results shown in Figure 4 also suggest that the block by MDL 12,330A is not voltage-dependent; voltage-dependent block would be associated with a curvature in the current-voltage relationship in the presence of the drug and such curvature was also not observed.

MDL 12,330A does not change the time constant of e.p.c. decay ($\tau_{e.p.c.}$). Figure 5a shows a comparison of e.p.cs evoked at the normal resting potential (−80 mV) in the presence and absence of 10 μM

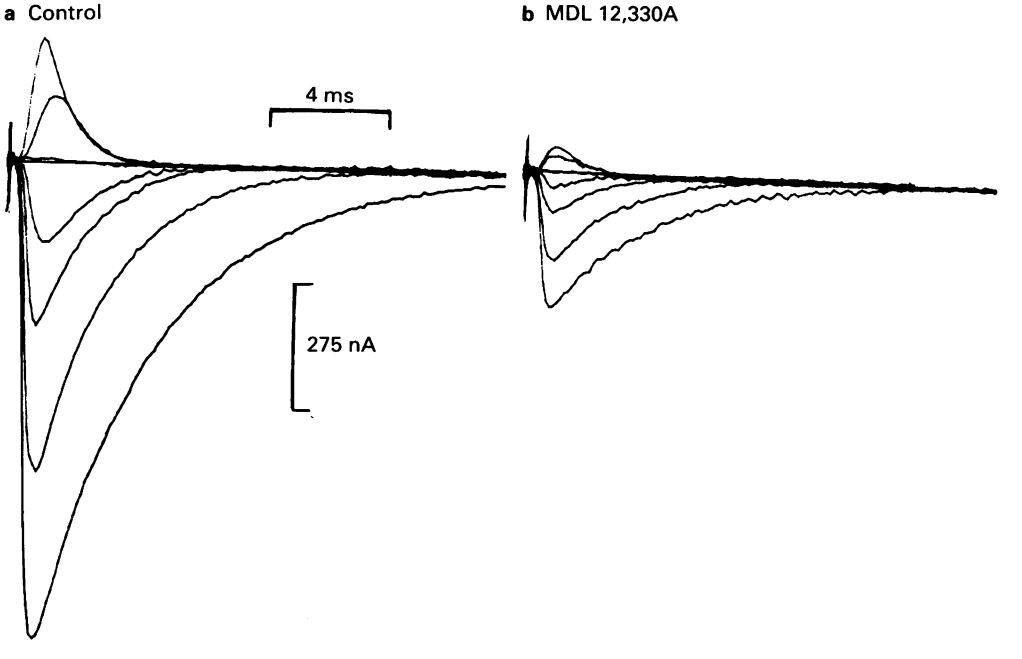

Figure 3 Representative endplate currents (e.p.cs) recorded at different membrane potentials (V_m) before (a) and after (b) 3 min of superfusion of a voltage clamped endplate with 10 μM MDL 12,330A. The values of V_m were (from the bottom upwards in mV): −140, −100, −60, −30, −10, +10, +30. Note the reversal potential was about −10 mV in both (a) and (b).

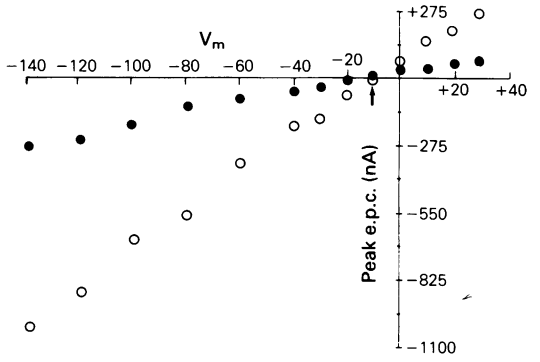

Figure 4 A plot of the peak endplate current (e.p.c.) against membrane potential (V_m) from the experiment of Figure 3. Note the absence of change in reversal potential (arrow) or the linearity of the current-voltage relationship. (○) Control records, (●) after 3 min in 10 μM MDL 12,330A.

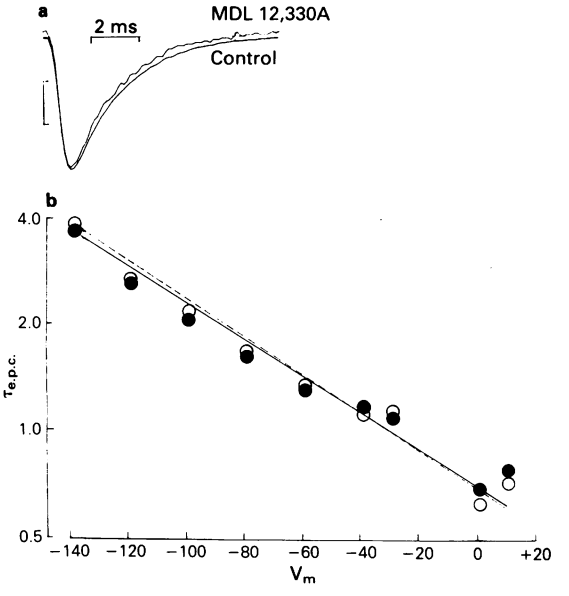

Figure 5 Absence of an effect of MDL 12,330A on the time constant of endplate current (e.p.c.) decay ($\tau_{e.p.c.}$). (a) Shows a control e.p.c. (− 521 nA, $\tau_{e.p.c.}$ = 1.7 ms) and a depressed e.p.c. in 10 μM MDL 12,330A (− 108 nA, $\tau_{e.p.c.}$ = 1.6 ms) that was scaled up to approximately the amplitude of the control e.p.c. Both e.p.cs were recorded at − 80 mV. Note the absence of significant change in $\tau_{e.p.c.}$. (b) Shows the absence of effect of 5 μM MDL 12,330A on the relationship between $\tau_{e.p.c.}$ and membrane potential (V_m). (○) Control records, (●) after 4 min in MDL 12,330A. Regression lines were drawn by computer.

MDL 12,330A. (Although the e.p.c. amplitude after 4 min in this drug concentration was depressed to 20% of the control value, the e.p.c. in the presence of MDL 12,330A was scaled upwards to near the control amplitude for illustrative purposes.) In this experiment and in four other similar experiments, the $\tau_{e.p.c.}$ determined at − 80 mV was not altered in the presence of MDL 12,330A. In several other experiments, $\tau_{e.p.c.}$ at different levels of V_m were compared in the presence and absence of drug (e.g. Figure 5b). In Figure 5b, note that the control relationship between $\tau_{e.p.c.}$ and V_m was unchanged after 4 min in 5 μM MDL 12,330A. The results shown in Figure 5 suggest that open channel blockade is not contributing to the inhibitory effects of MDL 12,330A, as according to current concepts, open channel block should be accompanied by pronounced changes in the kinetics of e.p.c. decay (e.g. Peper *et al.*, 1982). The postsynaptic effects of MDL 12,330A are thus reminiscent of those of an irreversible cholinoceptor blocker such as α-bungarotoxin (e.g. Peper *et al.*, 1982).

Discussion

These results demonstrate that the adenylate cyclase inhibitor MDL 12,330A exerts opposing actions on the frog neuromuscular junction; an enhancement of presynaptic function is accompanied by a profound reduction in the sensitivity of the endplate membrane to ACh. The presynaptic effect of this agent is consistent with the notion that a local increase in cyclic AMP concentration at a strategic cellular locus decreases ACh release (Silinsky, 1984). Specifically, certain agents that might be expected to inhibit cyclic AMP production increase ACh release. These include MDL 12,330A (Figure 1), P-site adenosine receptor agonists (Silinsky, 1980) and a stimulator of phosphodiesterases (e.g., imidazole; Ribeiro *et al.*, 1979). It has also been found recently that cyclic AMP delivered to the cytoplasm using lipid vesicles as a vehicle, decreases spontaneous and evoked quantal ACh release (Silinsky & Hirsh, unpublished observations).

These results add an additional element of controversy to the role of cyclic AMP in controlling ACh secretion as ACh release is also increased by a number of reagents expected to increase cyclic AMP (e.g., phosphodiesterase inhibitors and forskolin; Standaert & Dretchen, 1979; Silinsky, 1984 and unpublished observations). Exogenous cyclic AMP (as a dibutyryl derivative) has been found to increase ACh release in the rat diaphragm (Goldberg & Singer, 1969) and cat skeletal muscle (Standaert & Dretchen, 1979), to have no effect on ACh release under some conditions in the rat diaphragm (Miyamoto & Breckenridge, 1974) and even to inhibit ACh release during exposure but

increase release after removing the agent in the frog sympathetic ganglia (Kuba *et al.*, 1981; Kato *et al.*, 1985). All of these effects have been duplicated at the frog neuromuscular junction (unpublished observations), the particular result depending upon the composition of the bathing solution, the form of ACh release (spontaneous or evoked) and the particular cyclic AMP analogue (dibutyryl cyclic AMP, 8-bromocyclic AMP, or cyclic AMP in liposomes). Some of the effects of cyclic AMP derivatives may also be due to their actions on presynaptic adenosine receptors. Unfortunately, it is unlikely that an immediate resolution to the controversy is imminent as multiple cellular compartments within the experimentally inaccessible motor nerve endings may be involved in the complex effects of cyclic nucleotides on ACh release (see Dunwiddie & Hoffer, 1982 for discussion). For example, cyclic AMP-dependent phosphorylation of Ca storage sites could liberate Ca into the nerve terminal cytoplasm and increase ACh release.

In addition to inhibiting adenylate cyclase, MDL 12,330A at relatively high concentrations (0.1–1 mM) has been shown to exert additional actions, e.g. inhibition of ATPases and phosphodiesterase inhibition (Grupp *et al.*, 1980; Hunt & Evans, 1980). Based on the present findings it is unlikely that either of these effects are producing the electrophysiological changes at the neuromuscular junction. If MDL 12,330A inhibited the Na/K-ATPase, then a depolarization of the frog muscle membrane by over 20 mV would be predicted (Branisteanu *et al.*, 1979 – Table 1); this was never observed even during incubation with 0.1 mM concentrations of drug for many hours. While the effects of MDL 12,330A in increasing ACh release could be due to inhibition of ATPases, the concentrations of drug required for these effects would be higher than those employed in these experiments. If MDL 12,330A were inhibiting phosphodiesterases, then an increase in the inhibitory effect of adenosine would be expected rather than an occlusion (see Introduction in this paper and Figure 5 in Silinsky, 1984). Furthermore, most phosphodiesterase inhibitors do not decrease m.e.p.p. amplitude, and, when they do, the effect is associated with changes in e.p.c. kinetics (Silinsky & Vogel, unpublished observations); such changes were not observed in the presence of MDL 12,330A.

The decline in postsynaptic sensitivity to ACh caused by MDL 12,330A is most readily described as an irreversible, potential-independent block at the level of the inactive cholinoceptor. (Unfortunately, with the present methodology, the irreversibility of the drug precluded any comprehensive investigation of the relationship between extracellular [ACh] and inhibition by MDL 12,330A.) It is possible that the postsynaptic block could arise indirectly if MDL 12,330A inhibited adenylate cyclase located in the postsynaptic membrane. In this regard, cyclic AMP-dependent phosphorylation of a membrane protein constituent has been suggested to play a role in maintaining nicotinic receptors in a functional state (see e.g. Huganir & Greengard, 1983). While our present results with MDL 12,330A are not discordant with this view, in preliminary experiments we found that pretreatment of the tissue with concentrations of dibutyryl cyclic AMP sufficient to alter presynaptic function (4 mM) did not affect the inhibition produced by MDL 12,330A. It is still possible that a postjunctional membrane effect on a subunit of adenylate cyclase (apart from any changes in cyclic AMP concentrations) could be responsible for the inhibitory effects of MDL 12,330A, but a direct effect of this agent on the ACh receptor/ion channel cannot be excluded by these experiments. It appears that measurements of adenylate cyclase activity and cyclic AMP levels must be made before any firm conclusions can be reached.

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