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Antagonism of Neuromuscular Blocks by Germine Monoacetate

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The effects of germine monoacetate (GMA) on neuromuscular transmission were studied with intracellular microelectrodes in isolated sciatic nerve–sartorius muscle preparations of the frog (*Rana temporaria*). In muscle fibers treated with 5×10^{-7} g/ml of *d*-tubocurarine (*d*Tc) or 5×10^{-6} g/ml of succinylcholine (SCh), GMA, 10^{-6} g/ml, caused a repetitive endplate potential (EPP), followed by a repetitive action potential (AP), in response to indirect shock. In muscle fibers treated with 10^{-6} g/ml *d*Tc or 10^{-5} g/ml SCh, GMA did not cause any AP in response to indirect shock, although the EPP's were repetitive and augmented. After addition of GMA, the repetitive AP was evoked more rapidly by direct shock than by indirect shock in *d*Tc- or SCh-treated muscle. GMA had no effect on the amplitude of the EPP. An anticholinesterase-like effect of GMA was also ruled out. (Key words: Germine monoacetate; *d*-Tubocurarine; Succinylcholine; Neuromuscular transmission.)

GERMINE MONOACETATE (GMA) and germine diacetate (GDA), semisynthetic ester alkaloids of the veratrum family, have recently been proposed for use not only as therapeutic agents for myasthenia gravis, but also as antagonists of depolarizing and nondepolarizing neuromuscular blocking agents.[1–5] Flacke[6, 7] found that GMA and GDA enhanced the contractile tension evoked by indirect stimulation of muscle and also that evoked by direct stimulation of denervated or *d*-tubocurarine-treated muscle. In addition, Flacke[7] postulated that the primary site of action of GMA and GDA is the muscle membrane, since the increase in contractile tension was accompanied by repetitive electrical activity in the muscle membrane. Standaert and Detwiler[5] observed an additional effect of these agents in presynaptic nerve terminals.

The present study was designed to test the effects of GMA on neuromuscular transmission in frog muscle in the presence of *d*-tubocurarine (*d*Tc) or succinylcholine (SCh), particularly from the standpoint of interaction between drugs, and to determine whether GMA has an "anticholinesterase" action.

Materials and Methods

Sciatic nerve–sartorius muscle preparations of the frog (*Rana temporaria*) were used for all experiments. Each freshly isolated preparation was stretched to 130–140 per cent of its length and fixed in a bath of about 5 ml of Ringer's solution. The solution in the bath could be removed or added at a rate of 100–200 ml per hour. A change from one solution to another could be accomplished in 4–5 minutes.

The technique for intracellular recording of resting and action potentials and endplate potentials of individual muscle fibers was similar to that described by Fatt and Katz.[8] The glass capillary microelectrodes used were filled with 3 M KCl; resistances were between 10 and 30 M. For indirect stimulation of muscle fibers, the sciatic nerve was stimulated by a pair of silver electrodes 1 cm apart. For direct stimulation, a pair of glass microelectrodes was inserted into the muscle fiber intracellularly; one microelectrode was used to convey the current pulse and the other to record changes in membrane potential. The two microelectrodes were separated by less than 30 μ to allow measurement of the effective membrane resistance, which was calculated from a slope indicating the relationship between the intensity of the current and the steady level of polarization 25 msec after application of the current was begun.[8]

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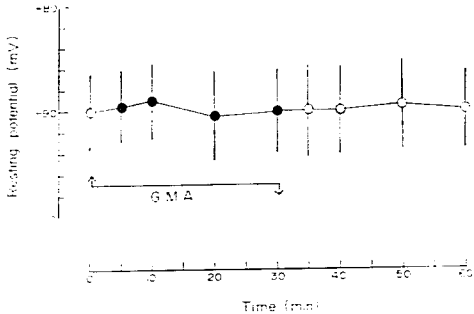

FIG. 1. Sequeneial changes in resting membrane potentials of muscle fiber. The muscle preparation was immersed in Ringer's solution (○) for 30 minutes, then in a solution containing 10^{-5} g/ml GMA (●) for 30 minutes, and again in Ringer's solution for 30 minutes (○). Each symbol and vertical bar indicate the mean and SD of results of 50 experiments in five muscles.

SOLUTIONS AND DRUGS

The Ringer's solution used had the following composition: NaCl 112.0 mM, KCl 2.0 mM, $CaCl_2$ 3.6 mM, NaH_2PO_4 1.0 mM, Na_2HPO_4 1.0 mM, maintained at *p*H 6.8. A sodium-deficient solution was made by replacing 82 mM NaCl with 147.6 mM sucrose. The temperature of the bath was kept constant at about 20 C.

The following drugs were used: germine monoacetate, 10^{-5} g/ml; *d*-tubocurarine, 10^{-6} and 5×10^{-7} g/ml; succinylcholine, 10^{-5} and 5×10^{-6} g/ml. All drugs were diluted in Ringer's solution. The stock solution of germine monoacetate at a concentration of 1 mg/ml was prepared by addition of diluted HCl (0.01 mM–0.001 mM).

Results

EFFECTS OF GMA ON RESTING MEMBRANE POTENTIAL AND EFFECTIVE RESISTANCE OF THE MUSCLE FIBER

Figure 1 represents the sequential changes in resting membrane potentials of muscle fibers bathed in Ringer's solution with and without 10^{-5} g/ml GMA. Neither resting membrane potential nor effective resistance was appreciably affected by GMA treatment for 30 minutes. Mean values are listed in table 1.

TABLE 1. Effects of GMA (10^{-5} g/ml) Treatment for 30 Minutes on Resting Potential and Effective Resistance of the Muscle Membrane*

	Resting Potential	Effective Resistance
Solution without GMA	89.7 ± 3.8 mV (50)	490 ± 144 KΩ (18)
GMA added to solution	90.1 ± 4.0 mV (49)	488 ± 124 KΩ (11)

* Values are means ± SD. Numbers in parentheses are numbers of muscle fibers tested.

EFFECTS OF GMA ON ACTION POTENTIALS OF MUSCLE FIBERS

Although GMA at a concentration of 10^{-5} g/ml had no conspicuous effect on resting membrane potential, it had a typical veratrine-like effect on the configuration of the action potentials (fig. 2). Soon (5 to 10 min) after addition of GMA, the action potential evoked by a single direct shock was accompanied by a prolonged and augmented negative after-potential, the half decay time of which was about 40 msec (fig. 2*B*).

GMA rendered the muscle fiber capable of firing repetitively in response to a single direct shock more than 10 minutes after treatment (fig. 2*C*). This veratrine-like effect of GMA remained for a fairly long time, being discernible even after washing out of the drug from the tissue for more than two hours (fig. 2*D*).

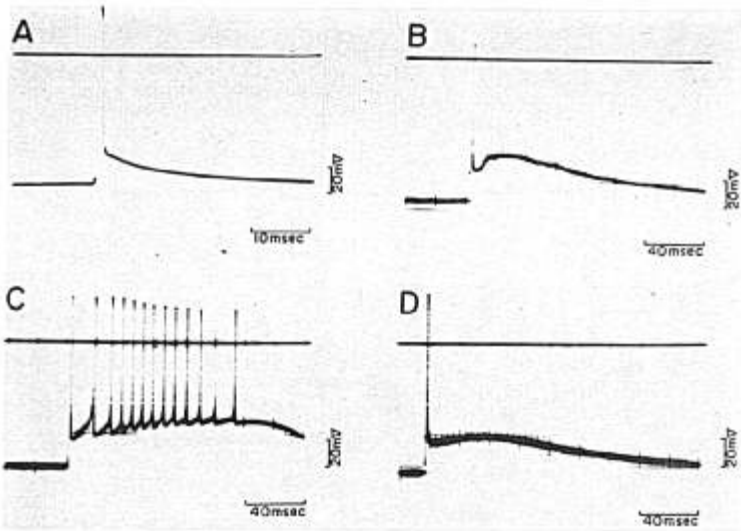

FIG. 2. Action potentials recorded from a single muscle fiber in response to direct stimulation. *A*, before GMA. *B*, 10 minutes after GMA, 10^{-6} g/ml, the action potential has a prolonged and augmented negative after-potential. *C*, 15 minutes after GMA treatment, repetitive firings can be seen. *D*, 120 minutes after washing out GMA, the action potential is similar to that in *B*.

EFFECT OF GMA ON NEUROMUSCULAR TRANSMISSION BLOCKED WITH *d*Tc

The preparation was first soaked in solution containing *d*Tc in a relatively high concentration (10^{-6} g/ml). *d*Tc at this concentration blocked the action potential of the muscle fiber, while the EPP in response to indirect electrical stimulation persisted after 10 minutes (fig. 3*A*). GMA, 10^{-5} g/ml, was then added to the bath containing *d*Tc (15 minutes after the initial application of *d*Tc). While no enhancing effect of GMA on amplitude of the EPP in response to indirect stimulation could be observed 10 minutes after addition of GMA, a repetitive action potential of a single muscle fiber could be elicited by direct stimulation (fig. 3*B*, *a* and *b*). More than 30 minutes after addition of GMA, a repetitive EPP appeared in response to indirect shock without initiating an action potential (fig. 3*C*). Amplitudes of EPP's showed characteristic augmentation and fading. The numbers of repetitive endplate potentials ranged from 15 to 22, believed to correspond to the numbers of repetitive action potentials occurring in the nerve terminals. In preparations exposed to high concentrations of *d*-tubocurarine, repetitive EPP's induced by GMA disappeared relatively rapidly (within 10 minutes) after washing out the drug (fig. 3*D*).

When *d*Tc was applied in a relatively low concentration (5×10^{-7} g/ml), blockade of transmission was established about 30 minutes after application (fig. 4*A*). Within 10 minutes after addition of GMA, the repetitive EPP in response to indirect shock was facilitated until the repetitive action potential was triggered in the muscle fiber (fig. 4*B*).

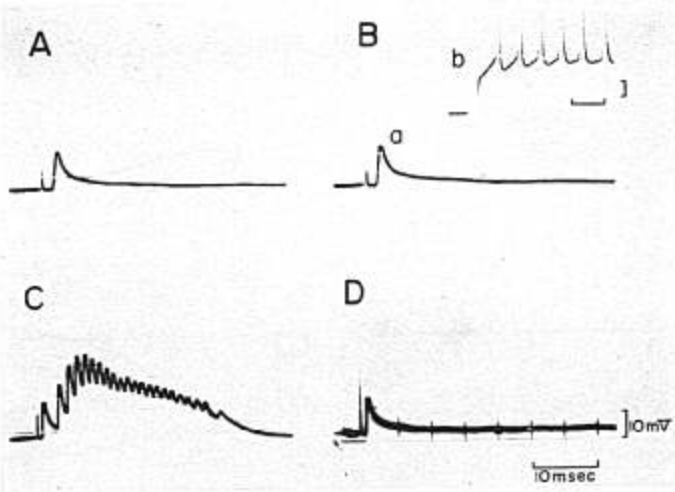

FIG. 3. Effects of GMA, 10^{-4} g/ml, on neuromuscular transmission blocked by *d*Tc, 10^{-4} g/ml. *A*, EPP in response to indirect electrical stimulation 30 minutes after application of *d*Tc. *B*, 15 minutes after addition of GMA to the *d*Tc-containing solution: *a*, EPP in response to indirect stimulation; *b*, repetitive firings of a muscle fiber in response to direct stimulation. *C*, 30 minutes after addition of GMA. Repetitive EPP in response to indirect stimulation shows augmentation and fading. *D*, 10 minutes after washing out GMA.

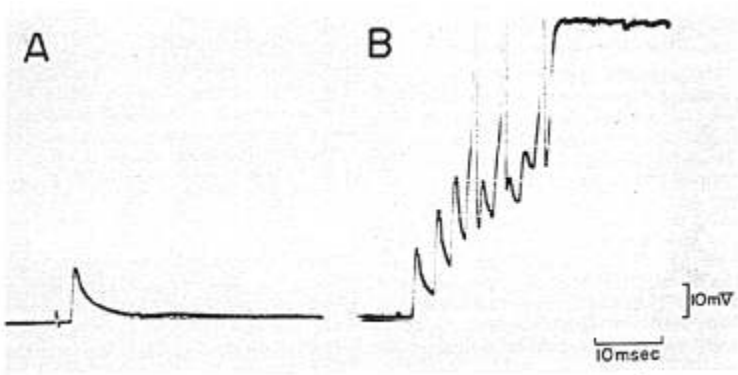

FIG. 4. Effects of GMA, 10^{-3} g/ml, on neuromuscular transmission blocked by *d*Tc (5×10^{-5} g/ml). *A*, 30 minutes after application of *d*Tc. *B*, 10 minutes after addition of GMA to the *d*Tc-containing solution. After recording three action potentials from the muscle fiber, the intracellular microelectrode was dislocated by the strong contraction of the whole muscle.

Effect of GMA on Neuromuscular Transmission Blocked with SCh

SCh at a concentration of 10^{-5} g/ml caused a rapid and progressive blockade of neuromuscular transmission (fig. 5*A–C*). The blockade was established 3 to 6 minutes after application. SCh in this concentration caused slight depolarization of the resting membrane potentials of endplate regions, amounting to 7.6 ± 2.1 mV (mean ±SD). Depolarization by SCh reached a maximum within 4 minutes after application; then the resting membrane potential was gradually repolarized due to desensitization, returning to control within 13 minutes after application of SCh. About 8 minutes after addition of GMA, 10^{-5} g/ml, to the solution, a repetitive EPP similar to that seen in preparations treated with relatively high doses of *d*Tc (fig. 3*C*) appeared (fig. 5*D*).

When SCh was applied at a concentration of 5×10^{-6} g/ml, blockade of transmission occurred about 10 minutes later in muscle fibers near the surface (fig. 6*A*). Complete blockade of the whole muscle took 30 to 50 minutes. Addition of GMA immediately after establishment of the blockade caused repetitive responses to a single indirect shock like those seen in the preparations exposed to low concentrations of *d*-tubocurarine (fig. 6*B*).

With increasing concentrations of *d*Tc or SCh in the bath, the amplitudes of EPP's di-

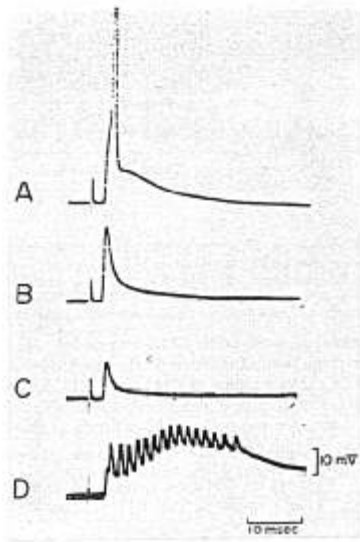

Fig. 5. Effects of GMA, 10^{-4} g/ml, on neuromuscular transmission blocked by SCh (10^{-5} g/ml). *A*, action potential triggered by indirect stimulation in Ringer's solution. *B*, 3 minutes after application of SCh. *C*, 5 minutes after application of SCh. *D*, 8 minutes after addition of GMA.

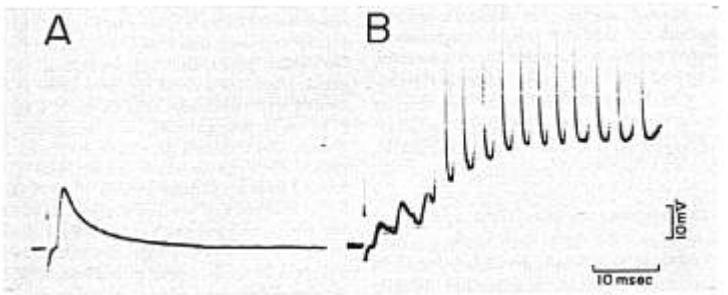

Fig. 6. Effects of GMA, 10^{-5} g/ml, on neuromuscular transmission blocked with SCh, 5×10^{-6} g/ml. *A*, EPP in response to indirect shock 10 minutes after application of SCh. *B*, 10 minutes after addition of GMA to SCh-containing solution. The repetitive EPP causes initiation of a repetitive action potential in the muscle fiber.

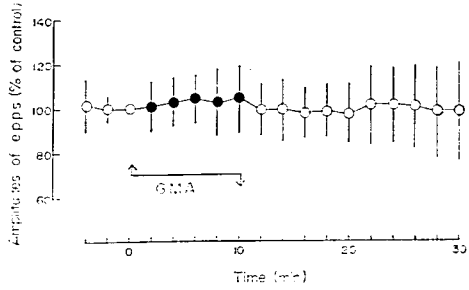

FIG. 7. Amplitudes of EPP's in a sodium-deficient solution. Measurements were begun 30 minutes after establishment of the neuromuscular block in the sodium-deficient solution (○). GMA, 10^{-5} g/ml, was then added; the preparation remained in the GMA-containing solution for 10 minutes (●), after which the drug was washed out with sodium-deficient solution for 20 minutes (○). Symbols indicate means ±SD of results of 11 experiments to determine amplitude of EPP's relative to control values, which were obtained just before addition of GMA in each experiment.

minished, even when GMA was added. The average rates of decrease in the amplitudes of EPP's after addition of GMA were 0.15 ± 0.11 mV/min in muscle fibers treated with *d*Tc for 30 minutes and 0.42 ± 0.28 mV/min in those treated with SCh for 10 minutes.

Effects of GMA on Amplitude and Time Course of the EPP in Low-sodium Solution

The effect of GMA, 10^{-5} g/ml, on the EPP was tested in muscle fibers in which neuromuscular transmission had been blocked by lowering the sodium concentration in the bath to about a fourth that in the Ringer's solution. From about 30 minutes after establishment of blockade of neuromuscular transmission, the amplitudes of EPP's remained constant for more than 30 minutes. As shown in figure 7, amplitude of the EPP was not significantly affected by GMA treatment for 10 minutes, being 20.2 ± 6.6 mV (mean ±SD) in the control period and 20.4 ± 5.9 mV 10 minutes after GMA treatment. Similarly, the decay-time course of the EPP was unaffected by GMA treatment for 10 minutes. The half decay times of EPP's in the control solution and in the solution containing GMA were 3.2 ± 0.5 and 3.3 ± 0.6 msec, respectively.

Treatment with GMA, 10^{-5} g/ml, for 15 to 30 minutes did not cause repetition of EPP's in four preparations tested in the sodium-deficient solution, although there was also a fifth preparation in which repetition occurred.

Discussion

The time course of the changes in membrane potentials following application of SCh was compatible with the observation of Thesleff[9] that depolarization produced by SCh lasts less than 15 minutes. The degree of depolarization by SCh was much less than values reported by others.[9, 10] However, Gissen and Nastuk[10] found that SCh in a concentration of about 10^{-5} g/ml depolarized the membrane potential to about −40 mV, an effect which lasted for at least 60 minutes. These differences may have resulted from the slow application of the drug by the perfusion technique and/or the weak acidity of the bathing solution (*p*H 6.8) in the present study.

In muscle fibers treated with relatively low concentrations of *d*Tc or SCh, GMA caused repetitive EPP's in response to single indirect stimuli and augmented their amplitudes until the action potentials were repetitively triggered (figs. 4*B* and 6*B*). In muscle fibers treated with relatively high doses of *d*Tc or SCh, no action potential was triggered, although GMA caused repetitive EPP's in response to single indirect stimuli and augmented their amplitudes as well (figs. 3*C* and 5*D*). Therefore, it seemed worthwhile to find out whether there was any significant difference between low and high concentrations of *d*Tc or SCh with respect to facilitation of repetitive EPP's by GMA. As summarized in table 2, there was no significant difference ($P > 0.5$) as regards the degrees of facilitation of the second or third EPP's by GMA.

TABLE 2. Amplitudes of the First EPP's (E_1) and Degrees of Facilitation of the Second (E_2) and Third (E_3) EPP's in a Series of EPP's Caused by Application of GMA to Muscle Fibers Treated with *d*Tc or SCh for 3 to 30 Minutes*

Solution	E_1 (mV)	E_2 (ff)	E_3 (ff)	n	Action Potential
A 1) *d*Tc (10^{-4} g/ml) + GMA	6.6 ± 2.1	0.34 ± 0.17	0.74 ± 0.32	9	(−)
2) SCh (10^{-5} g/ml) + GMA	6.0 ± 3.5	0.23 ± 0.17	0.62 ± 0.32	6	(−)
B 3) *d*Tc (5×10^{-7} g/ml) + GMA	15.6 ± 4.0	0.46 ± 0.34	0.92 ± 0.54	6	(+)
4) SCh (5×10^{-6} g/ml) + GMA	13.8 ± 3.9	0.41 ± 0.18	0.63 ± 0.20	7	(+)

* Values are means ± SD.

† f is the degree of facilitation defined by an equation, $f = (v - v_0)/v_0$, where v is the amplitude of the second or third EPP, and v_0, the amplitude of the first EPP (see Mallart and Martin[9]). Comparing the values obtained in muscle preparations treated with high doses of *d*Tc or SCh (A) with those obtained in preparations treated with low doses of *d*Tc or SCh (B), no significant difference was found except in the amplitudes of the first EPP's (E_1). Whether or not an action potential was initiated in response to indirect shock in each solution is expressed by "(+)" or "(−)" at the last column.

There was a significant difference ($P < 0.01$) between the amplitudes of the first EPP's in *d*Tc- and SCh-treated muscles. The degree of facilitation found in the present study was in agreement with that found in the Mg-blocked or *d*-tubocurarine-treated endplate of the frog by Mallart and Martin.[11, 12] These results indicate that GMA does not affect release of transmitter substance during repetitive firing of nerve endings. Furthermore, that GMA did not increase the progressively diminishing amplitudes of single EPP's treated with *d*Tc or SCh supports this thesis. There is evidence that any drug which acts on the transmitter substance modifies the amplitude and time course of the EPP. Since the amplitude and half decay time of the EPP were not affected by GMA treatment in the sodium-deficient solution, an "anticholinesterase-like" effect of GMA could be ruled out.[13]

Recently, Detwiler[14] noticed that the production of repetitive nerve activity by GMA is not a consequence of increased release of transmitter substance or intensification or prolongation of transmitter action. There are also reports[1, 15] that veratrine does not affect transmitter release in the Mg-blocked rat diaphragm, while in the intercostal muscle of the myasthenic patient, both GMA and veratrine increase transmitter release during tetanic nerve stimulation.

There is ample evidence for the ionic mechanism underlying veratrine action on excitable membrane, *i.e.*, the agent prolongs the inward-going Na^+ current during activation of the membrane and consequently causes repetitive action potentials with marked, prolonged negative after-potentials.[16] It is likely that the same mechanism may operate in the action of GMA.[5-7] This hypothesis might be supported by the present finding that treatment with GMA for 15–30 minutes did not cause repetition of EPP's in sodium-deficient solution (although there was an exception). However, a more detailed explanation of ionic mechanism underlying GMA action was not provided by the results of the present experiment. Veratrine at a concentration of 10^{-5} g/ml depolarized rat muscle fiber and reduced effective membrane resistance.[17] It was demonstrated in the present study that GMA at the same concentration has no or little effect on the resting membrane. Repetitive firing caused by GMA in the *d*Tc- or SCh-treated muscle developed more rapidly in response to direct stimulation than in response to indirect stimulation (fig. 3*B*). This may have resulted from the difference between the sensitivities to GMA of pre- and postjunctional membranes.

Most drugs that affect neuromuscular transmission are believed to act by blocking, enhancing, or replacing the effects of ACh. In contrast, in clinical doses GMA lacks a depolarizing effect.[14] A cholinesterase-inhibiting action was not found in this study, nor does GMA have a cholinesterase-like effect[5] or an

anti-acetylcholine action.[14] The present study confirms that the essential feature of GMA's action on neuromuscular transmission is the conversion of a single response into a train of repetitive action potentials, although the mechanism for the generation of repetitive activity by GMA is not known. Thus, GMA greatly increases the strength of contractions of skeletal muscle,[2, 5, 6] is useful for treating myasthenia gravis,[1] and antagonizes both depolarizing and nondepolarizing neuromuscular blocks.[2, 5]

One reason GMA is still not used clinically may be that it lacks effects on ACh, as mentioned above. Nevertheless, the strong effect of GMA on neuromuscular transmission and its antagonism of both depolarizing and nondepolarizing neuromuscular blocks should be evaluated more precisely from the standpoint of clinical anesthesia.

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References

1. Hofmann WW: Newer drugs for myasthenia gravis; a microphysiologic study of effects. J Pharmacol Exp Ther 160:349–359, 1968
2. Flacke W, Katz RL, Flacke JW, et al: Germine diacetate as an antagonist of depolarizing and nondepolarizing neuromuscular agents in man. ANESTHESIOLOGY 29:850–854, 1968
3. Foldes FF, Brodman EB, Kranzler HN: The effects of germine diacetate on the rat phrenic nerve–diaphragm preparation. ANESTHESIOLOGY 31:522–531, 1969
4. Katz RL, Flacke W: Neuromuscular effects of germine diacetate in cat and man. Progress in Anaesthesiology; Proceedings of the Fourth World Congress of Anaesthesiologists, London, September 1968, Excerpta Medica, 1970, pp 437–442
5. Standaert FG, Detwiler PB: The neuromuscular pharmacology of germine-3-acetate and germine-3,16-diacetate. J Pharmacol Exp Ther 171:233–241, 1970
6. Flacke W: Studies on veratrum alkaloids. 33. The action of some esters of germine with acetic acid on the sartorius muscle of the frog. J Pharmacol Exp Ther 137:62–69, 1962
7. Flacke W: Studies on veratrum alkaloids. 36. The action of germine monoacetate and germine diacetate on mammalian skeletal muscle. J Pharmacol Exp Ther 141:230–231, 1963
8. Fatt P, Katz B: An analysis of the end-plate potential recorded with an intracellular electrode. J Physiol 115:320–370, 1951
9. Thesleff S: The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine. Acta Physiol Scand 34:218–231, 1955
10. Gissen AJ, Nastuk WL: Succinylcholine and decamethonium. ANESTHESIOLOGY 33:611–618, 1970
11. Mallart A, Martin AR: An analysis of facilitation of transmitter release at the neuromuscular junction of the frog. J Physiol 193:679–694, 1967
12. Mallart A, Martin AR: The relation between quantum content and facilitation at the neuromuscular junction of the frog. J Physiol 196:593–604, 1968
13. Kordas M: A study of the end-plate potential in sodium deficient solution. J Physiol 198:81–90, 1968
14. Detwiler PB: The effects of germine-3-acetate on neuromuscular transmission. J Pharmacol Exp Ther 180:244–254, 1972
15. Hofmann WW, Parsons RW, Feigen GA: Effects of temperature and drugs on mammalian motor nerve terminals. Am J Physiol 211:135–140, 1966
16. Ulbricht W: The effect of veratridine on excitable membranes of nerve and muscle. Ergeb Physiol 61:18–71, 1969
17. Yonemura K: Depolarizations produced by veratrine in rat skeletal muscle fibers. Kumamoto Med J 23:41–55, 1970