Low-frequency Neuromuscular Depression Is a Consequence of a Reduction in Nerve Terminal Ca^{2+} Currents at Mammalian Motor Nerve Endings

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ABSTRACT

Background: The decline in voluntary muscle contraction during low-frequency nerve stimulation is used clinically to assess the type and degree of neuromuscular block. The mechanism underlying this depression is unknown.

Methods: Simultaneous electrophysiological measurements of neurotransmitter release and prejunctional Ca^{2+} currents were made at mouse neuromuscular junctions to evaluate the hypothesis that decreases in nerve terminal Ca^{2+} currents are responsible for low-frequency depression.

Results: Under conditions generally used to measure Ca^{2+} currents at the neuromuscular junction, increasing the frequency of nerve stimulation briefly from 0.017 to 0.1–1 Hz caused a simultaneous reduction in the release of the neurotransmitter acetylcholine to $52.2 \pm 4.4\%$ of control and the Ca^{2+} current peak to $75.4 \pm 2.0\%$ of control ($P < 0.001$, n = 5 experiments for both measurements, mean ± SEM for all data). In conditions used for train-of-four monitoring (4 stimuli, 2 Hz), neurotransmitter release declined to $42.0 \pm 1.0\%$ of control and the Ca^{2+} current peak declined to $75.8 \pm 3.3\%$ of control between the first and fourth stimulus ($P < 0.001$, n = 7 experiments for both measurements). Depression in acetylcholine release during train-of-four protocols also occurred in the absence of neuromuscular-blocking drugs.

Discussion: The results demonstrate that neuromuscular depression during train-of-four monitoring is due to a decline in nerve terminal Ca^{2+} currents, hence reducing the release of acetylcholine. As similar processes may come into

What We Already Know about This Topic

- Neurotransmitter release and voluntary muscle contraction decline during low-frequency nerve stimulation but the mechanism underlying this neuromuscular depression is unknown

What This Article Tells Us That Is New

- Neuromuscular depression during train-of-four monitoring is due to a decline in nerve terminal Ca^{2+} currents, hence reducing the release of acetylcholine, which may have relevance for development of agents that antagonize the decline in Ca^{2+} currents for use in debilitating diseases affecting neuromuscular transmission

play at higher stimulation frequencies, agents that antagonize the decline in Ca^{2+} currents could be used to treat conditions in which neuromuscular depression can be debilitating.

THE efficiency of skeletal neuromuscular transmission is limited by the neuromuscular depression that ensues even with brief activation of the motor nerve. Neuromuscular depression is due to a decline in the release of the neurotransmitter acetylcholine with repetitive stimulation.[1–3] As a consequence of both the large amounts of acetylcholine available for release and the surfeit of acetylcholine receptors, the normal neuromuscular junction generally has a high functional reserve for the production of muscle twitches. Hence, in the absence of neuromuscular disease, mild neuromuscular depression is not generally manifested as a decline in twitch tension of the muscle. In contrast, this depression of acetylcholine release can be extremely debilitating in neuromuscular diseases such as myasthenia gravis, where the functional reserve of the neuromuscular junction is encroached upon by the destruction of acetylcholine receptors in the muscle.[4]

The decline in neuromuscular transmission is often used by anesthesiologists to assess the type and degree of neuromuscular blockade produced by neuromuscular-blocking drugs[5–8] and by neurologists for the differential diagnosis of diseases of the neuromuscular junction.[9] Anesthesiologists commonly use train-of-four (TOF) monitoring to assess neuromuscular function during anesthesia. In this pattern of stimulation, four consecutive stimuli at a frequency of 2 Hz are applied to the motor nerve and the amplitude of the fourth muscle twitch is compared with that of the first (control) twitch. TOF monitoring is also used to assess

the presence of residual neuromuscular block after surgical procedures as well as the reversal of block after drug administration.[5–8]

Despite the widespread acceptance that neuromuscular depression is due to a decrease in acetylcholine release, the cellular mechanism responsible for this phenomenon is still unclear. It is commonly believed that the low-frequency rundown in tension measurements during TOF monitoring is due to a depletion of the immediate available store of acetylcholine in nerve endings.[6,7] For example, it has been stated that: "TOF … is based on the concept that acetylcholine is depleted by successive stimuli."[7] However, the mammalian neuromuscular junction has an estimated pool of 69,000–276,000 acetylcholine-containing synaptic vesicles[10] even with greater pools of vesicles observed at amphibian neuromuscular junctions.[11] Given that only approximately 36–100 vesicles release their acetylcholine content in response to a nerve impulse at mammalian neuromuscular junctions[12,13]; it is possible that events in the nerve terminal other than a depletion of acetylcholine could be responsible for this low-frequency depression that occurs during TOF monitoring. In this regard, a component of the depression of acetylcholine release from rat motor nerve endings is likely to involve a decrease in the probability of acetylcholine secretion.[1] As the probability of secretion is related to the extracellular Ca^{2+} concentrations,[2] one possibility to consider is that the depression during TOF monitoring occurs as a consequence of a reduction in the nerve terminal Ca^{2+} current that couples depolarization to the secretion of acetylcholine. To test this hypothesis, simultaneous electrophysiological measurements were made of acetylcholine release (end-plate potentials [EPPs]) and nerve terminal Ca^{2+} currents[14,15] to determine the mechanism of this low-frequency neuromuscular depression.

Materials and Methods

General Description of Preparations and Recording Methods

Isolated murine phrenic nerve-hemidiaphragm preparations were used in accordance with the guidelines of the Northwestern University Animal Care and Use Committee (Northwestern University, Chicago, Illinois) and the National Institutes of Health (Bethesda, MD). Mice (B6129F2J, 20–30 g in weight) were humanely anesthetized with isoflurane and exsanguinated when unresponsive to tactile stimulation. Isolated phrenic nerve-hemidiaphragm preparations were pinned in a recording chamber and superfused with physiological saline solution at room temperature (21°–23°C) using a peristaltic pump (3.0 ml/min). In the rodent diaphragm, the level of evoked release is essentially unchanged in the temperature range between 21°C and 39°C (see the study by Hubbard *et al.*).[16]

Electrophysiological recordings were made using an Axoclamp 2A, DigiData 1200 or TL-125 interfaces and pCLAMP software (Axon Instruments, Inc., Sunnyvale,

CA) installed in a microcomputer. Electrophysiological waveforms were averaged by computer, and ASCII files from the pCLAMP recordings were imported to Sigma Plot (Systat Software, SPSS Inc., Chicago, IL) and then exported to Microsoft PowerPoint (Microsoft Corporation, Chicago, IL) for lettering.

Specific Recording Methods and Solutions

Electrophysiological recordings of voltage changes in the perineural space produced by Ca^{2+} entry *via* voltage-gated Ca^{2+} channels were made using the perineural recording technique described previously.[14,15] In brief, the perineural recording electrode, which when filled with normal Ringer's solution had resistances of 3–10 MΩ, was first positioned under visual control near small axon bundles at the ends of the myelin sheaths. Intracellular recordings of EPPs were made from end-plate regions of skeletal muscle simultaneously with perineural recordings in many experiments.[14,15] The intracellular recording electrode was filled with 3 M KCl (resistances in the range of 10–20 MΩ) and positioned within 50 μ of the perineural recording electrode.

The standard control physiological saline solution used to treat the preparations at the initial part of all the experiments contained: NaCl, 137 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 2 mM; and dextrose, 11 mM, buffered with 30 mM HEPES (pH 7.2–7.4) and gassed with 100% oxygen. The solutions used for simultaneous measurements of Ca^{2+} currents at high transmitter outputs (solutions used normally to measure Ca^{2+} currents) had the K^+ channel blockers 3,4,-diaminopyridine (300 μM) and tetraethylammonium chloride (10 mM) added to the control physiological solution to expose the underlying Ca^{2+} currents.

To record Ca^{2+} currents and EPPs at more normal levels of acetylcholine release, the ionic composition of the solution was changed to contain lower Ca^{2+} concentrations (0.7 mM of Ca^{2+}) and reduced concentrations of the potassium channel blockers 3,4,-diaminopyridine (100 μM) and tetraethylammonium (250 μM; *physiological Ca^{2+} current solution*; see the study by Silinsky).[15] In most experiments, D-tubocurarine chloride (7–20 μM) was used to reduce the EPPs below threshold for action potential generation. All chemicals were purchased from the Sigma Chemical Company, St. Louis, Missouri, except for vecuronium chloride which was obtained from USP, Rockville, MD.

Justification of the Perineural Recording Method

The perineural waveforms shown in the text are in actuality voltage changes produced by current flow across the resistance of the perineural sheath. The size of these currents is directly proportional to the difference in voltage between the nodes of Ranvier and the nerve endings. The inward Ca^{2+} current localized in the nerve terminals produces a proportional current in the perineural space, a current that flows back from the nerve ending to the recording site, where it is measured as an upward-going deflection. Thus, although they are not
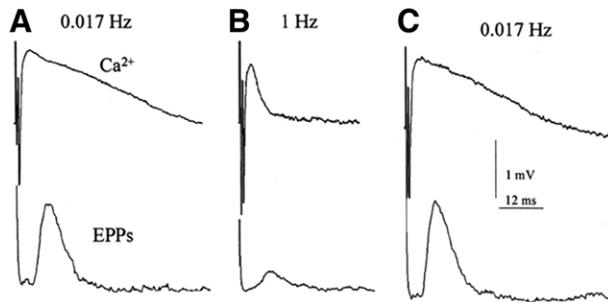
Fig. 1. Simultaneous depression and recovery of both nerve terminal Ca²⁺ currents (Ca²⁺) and end-plate potentials (EPPs). Each record is the average response to five stimuli. (*A*) Shows the control responses to very low-frequency nerve stimulation (0.017 Hz). (*B*) Shows that increasing the frequency of stimulation to 1 Hz causes a simultaneous reduction in both Ca²⁺ current and EPPs (stable levels were achieved within 4 stimuli). (*C*) Shows the recovery of both EPPs and Ca²⁺ currents approximately 4 min after the stimulus frequency was reduced to 0.017 Hz. The bathing solutions contained normal physiological salt solutions and the potassium channel blockers, such as 3,4,-diaminopyridine (300 μM) and tetraethylammonium (10 mM), to expose the underlying Ca²⁺ currents. Tubocurarine was used to reduce EPPs below threshold. Even after the complete block of Ca²⁺ currents by Cd²⁺, a residual outward current is present (seen as an upward deflection in *B*. This residual current has not been characterized; it has been hypothesized to be a residual Na⁺ current associated with the action potential or a current that passively repolarizes the part of nerve ending under the recording electrode.[14,15] The downward spike that precedes the Ca²⁺ components reflects the Na⁺ current associated with the propagating action potential in the nerve ending (see the studies by Redman *et al.* and Silinsky[14,15] and also fig. 3).

Fig. 2. Comparisons of the effects of depression produced by increased frequency of nerve stimulation with Ca²⁺ channel block by Cd²⁺ and the presynaptic modulator adenosine. Note that similar inhibition of release (end-plate potentials [EPPs]) is produced by similar inhibition of Ca²⁺ currents under three conditions. For depression produced by increasing the frequency of nerve stimulation, a frequency between 0.1 and 1 Hz was used to obtain stable EPPs that were approximately 50% of the control level. Cd²⁺ (10–20 μM) and adenosine (10 mM, which is a supramaximal concentration) were used for comparison with other results. The bathing solution was the same composition as that used for figure 1. For further details see text. Data for Cd²⁺ and adenosine were taken from the review by Silinsky,[15] in which the frequency of stimulation was 0.017 Hz.

strictly membrane ionic currents, the Ca²⁺ component of the perineural waveform is directly linked to highly localized nerve terminal membrane Ca²⁺ conductance changes. The method is accurate enough to detect changes in Ca²⁺ currents associated with the changes in acetylcholine release during TOF depression. Furthermore, this method allows for the simultaneous monitoring of acetylcholine release and Ca²⁺ currents at the same end-plate at normal levels of acetylcholine secretion (for complete justification, see studies by Redman and Silinsky).[14,15] It should be noted that the perineural electrode records from a small number of axons and not a single terminal. These recordings reflect the inverted images of the localized ionic currents that emanate from a single nerve terminal and with an increased signal to noise ratio because of the contribution from multiple axons in the region. For the sake of convenience, therefore, these waveforms will be termed presynaptic Ca²⁺ currents. Perineural waveforms were measured from the baseline to the peak of the current and presented in units of millivolt for the aforementioned reasons described. Stability of the recording electrode was assessed by monitoring the Na⁺ component of the perineural waveform; results were discarded if the magnitude of this component changed as such a change is indicative of
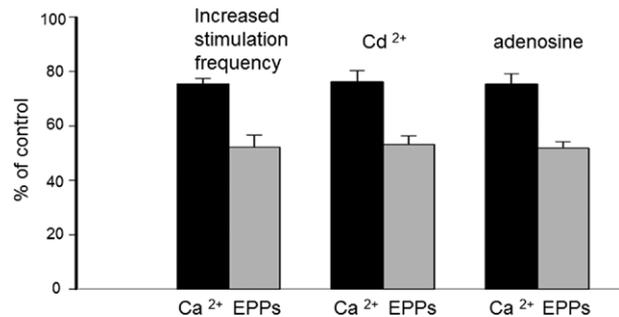
a change in electrode position. Success rates in these experiments are approximately 48% (n = 27 experiments).[15]

Quantifying Acetylcholine Release and Statistical Methods
Performing electrophysiology experiments on acetylcholine release *in vitro* provides a statistical advantage in that each neuromuscular junction serves as its own control. When experiments are made at normal or increased level of acetylcholine secretion, only modest numbers of EPPs need to be averaged to obtain statistically significant differences between the control and the experimental condition (for specific details, see the study by Silinsky).[17] In this regard, statistical significance is described both for each individual experiment and for the replicate experiments. Replication of experiments under the same conditions were made in different preparations (*i.e.*, n = x experiments indicates x replicate experiments performed in x different preparations).

After testing the data for normality, statistical comparisons between control and treated cells were made using the appropriate parametric or nonparametric statistical analysis. Normality was tested using the Shapiro–Wilk test. In most instances, as the data were normally distributed and of equivalent variance, statistical comparisons between the control and treatment (*e.g.*, between the response to the first and fourth stimulus) were made using a *t* test (two-tailed). The vast majority of experiments were analyzed in this manner. In two experiments, the data did not pass the normality test. In these experiments, the Mann–Whitney rank sum test was used. In the instance in which more than two groups were compared for normally distributed data, an ANOVA
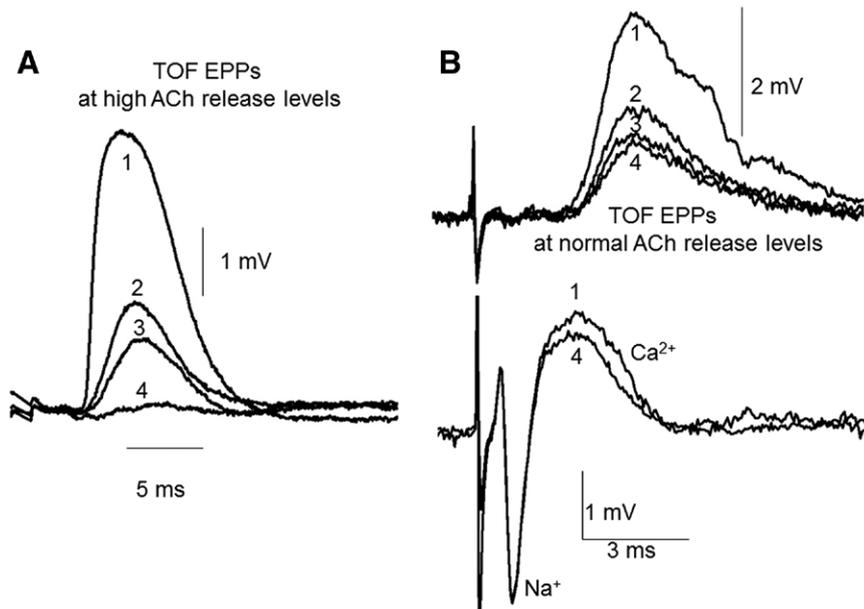
Fig. 3. Effects of train-of-four (TOF) stimulation at high release levels (*A*) and at more normal levels of acetylcholine release (*B*). Note that the steep rundown of acetylcholine release reflected as end-plate potentials (EPPs) at high release levels when compared with the normal. Traces in *A* were consecutively recorded. Solutions used for *A* were the same as figures 1 and 2. The solution used for *B*, termed *physiological Ca²⁺ current solution*, contained reduced Ca²⁺ concentrations (0.7 mm), reduced concentrations of potassium channel blockers (100 μm 3,4,-diaminopyridine and 250 μm tetraethylammonium) and tubocurarine to reduce EPPs below threshold for action potentials.[15] Traces in *B* were the averaged records of four consecutive TOF repeats (TOF ratio = 0.39). The TOF ratio for Ca²⁺ currents in this experiment was 0.78 (*P* = 0.03). *B* also shows the Na⁺ component of the propagating action potential. For further details see text.

was followed by multiple comparisons using the Bonferroni inequality if needed.[18] Data are presented as the mean ± SEM. Statistical analyses were performed using the Sigma Plot and Sigma Stat software packages (Systat Software, SPSS Inc.). For more complete details and justification, see the studies by Redman and Silinsky.[14,15,17]
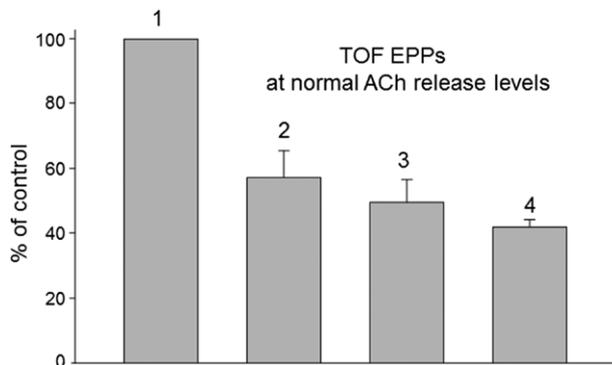

Fig. 4. Average train-of-four (TOF) ratios for end-plate potentials (EPPs) at normal acetylcholine outputs in the presence of neuromuscular blockade. The average data from all five experiments made in physiological Ca²⁺ current solution containing tubocurarine are shown. The number 1 refers to the response to the first stimulus with 2, 3, and 4 referring to responses to subsequent stimuli. The average TOF ratio for EPPs was 0.42 ± 0.05 (*P* < 0.001, n = 5 experiments). The decrease in average Ca²⁺ currents such as depicted in figure 3B was highly significant (see text for further details).

The mean number of acetylcholine quanta released was determined either directly, from the ratio of the mean EPPs to the mean miniature EPPs amplitude, or by using the tubocurarine method (discussed later in the article and the studies by Hirsh *et al.* and Silinsky).[12,17] To determine the mean number of acetylcholine quanta released by a nerve impulse (*M*) in *physiological Ca²⁺ current solution*, the tubocurarine method was used in conjunction with the following equation (Equation 1):

$$M = \left(\frac{\text{Mean EPP amplitude in tubocurarine}}{\substack{\text{Mean miniature EPP amplitude in} \\ \text{tubocurarine free solution}}} \right) \times (1 + K_{TC}[\text{tubocurarine}])$$

where K_{TC} is the equilibrium affinity constant for tubocurarine (2.6 μM⁻¹)—for justification, see the studies by Hirsh *et al.* and Silinsky.[12,17]

The motor nerve was stimulated at 0.05–0.1 Hz to preclude neuromuscular depression and allow for accurate assessments of the basal level of evoked acetylcholine release in this solution. Equation 1 was also used to make a rough estimate of the number of quanta released in the solutions used in figures 1, 2, and 3A as follows: the average miniature EPP amplitude in the absence of tubocurarine (2 mV)
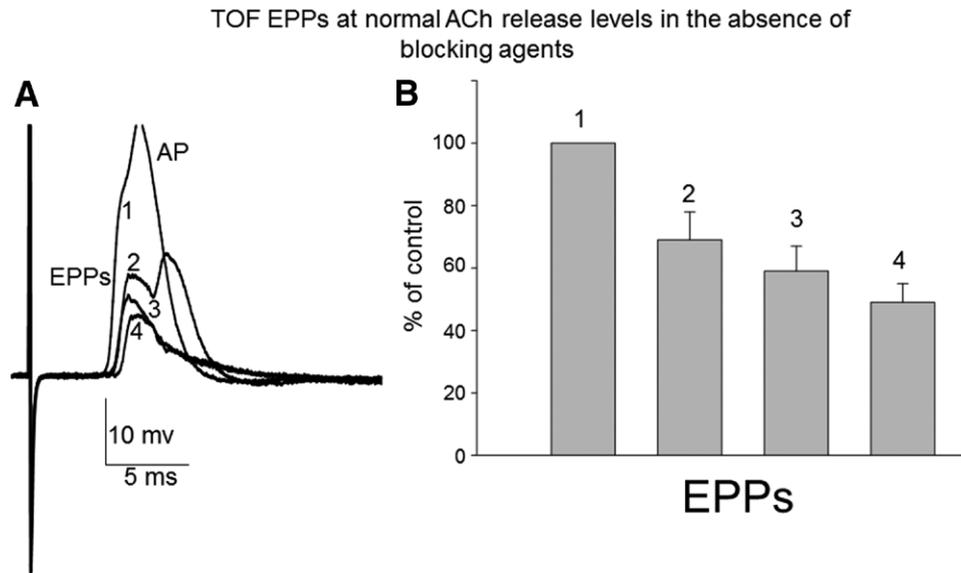
TOF EPPs at normal ACh release levels in the absence of blocking agents

Fig. 5. End-plate potentials (EPPs) during train-of-four (TOF) stimulation at normal levels of acetylcholine release in the absence of blocking drugs. Normal physiological saline solutions were used. (*A*) Shows raw data from an experiment in which the average number of quanta released was 21 as measured directly from the ratio of the mean EPP amplitude to the miniature EPP amplitudes. (*B*) Shows the average data from all four preparations. As described in the text, no differences were observed between the TOF ratio in the presence of the neuromuscular-blocking drugs such as tubocurarine or vecuronium (these experiments were made in physiological Ca²⁺current solution) and those made in the absence of blocking drugs in normal bathing solutions.

was normalized by the equilibrium dissociation constant of tetraethylammonium as a blocker to block nicotinic receptors at the mouse neuromuscular junction (1 mM).[19] In the presence of 20 μM tubocurarine and 10 mM tetraethylammonium, several experiments revealed EPPs of approximately 8 mV. Hence substituting these values into equation 1:

$$M = (8\ \text{mV} / 0.18\ \text{mV})(1 + 20 \mu M \times 2.6\ \mu M^{-1})$$
$$= 2{,}332\ \text{acetylcholine quanta per nerve impulse}$$

Results

Depression of Ca²⁺ Currents and Neurotransmitter Release in Normal Bathing Solutions Containing Potassium Channel Blockers

Figure 1 shows a typical experiment made under conditions generally used to measure Ca²⁺ currents at the skeletal neuromuscular junction, namely normal physiological bathing solutions containing high concentrations of K⁺ channel blockers to unmask the underlying Ca²⁺ current.[15] Under these conditions, as the frequency of stimulation was increased from 0.017 Hz (A) to 1 Hz (B), the average Ca²⁺ current peak (upper traces) declined in parallel with the electrophysiological correlates of acetylcholine release (EPPs; fig. 1; lower traces; *P* < 0.001). This effect was fully reversible after lowering the rate of stimulation back to 0.017 Hz (fig. 1C; for further details of the Ca²⁺ current waveform; see figure legend and Methods and Materials). In order to determine whether the observed reductions in Ca²⁺ currents were sufficient to account for the level of neuromuscular

depression observed in figure 1, experiments such as that shown in figure 1 were made by increasing the frequency of nerve stimulation from 0.017 to 0.1–1 Hz and measuring Ca²⁺ currents when acetylcholine release was decreased to a stable level of approximately 50% of the control level. Figure 2 (first 2 bars) summarizes the data; note that the mean Ca²⁺ current peak was reduced to approximately 75.4 ± 2.0% of control (*P* < 0.001, n = 5 experiments, mean ± SEM for all data) when the mean EPP amplitude was reduced to 52.2 ± 4.4% of control (*P* < 0.001, n = 5 experiments) by increasing the frequency of nerve stimulation.

Figure 2 also depicts data for two other agents demonstrated to inhibit acetylcholine release as a consequence of a reduction in Ca²⁺ currents, namely the inorganic Ca²⁺ channel blocker Cd²⁺ (which blocks Ca²⁺ currents directly) and the presynaptic modulator adenosine (which reduces Ca²⁺ currents indirectly *via* activation of G-protein coupled A₁ adenosine receptors).[15] All the three methods for reducing EPP to approximately 50% of the control level produced similar decreases in Ca²⁺ currents. Specifically, figure 2 shows that when decreases in EPPs to approximate 50% of the control level were produced by 0.1–1 Hz frequencies of stimulation, by Cd²⁺ (EPPs declined to 53.2 ± 3.2% of control, n = 5 experiments) or by adenosine (EPPs declined to 51.8 ± 2.3% of control, n = 5 experiments); all three methods of reducing EPPs to similar levels produced similar decreases in Ca²⁺ currents. Thus the Ca²⁺ current after the application of Cd²⁺ declined to 76.2 ± 4.1% of control and after the application of adenosine to 75.4 ± 3.9% of control. ANOVA revealed no significant differences among the groups for the decrement

in EPPs ($P = 0.95$) or Ca^{2+} currents ($P = 0.98$). Under these conditions, it thus seems that decreases in acetylcholine release during repetitive nerve stimulation can be fully explained by a reduction in prejunctional Ca^{2+} currents.

The experiments showed in figures 1 and 2 were performed under conditions that allow for stable EPPs to be recorded simultaneously with substantial Ca^{2+} currents in normal physiological salt solutions but are made under nonphysiological conditions of increased levels of neurotransmitter release.[11–14] For example, it is estimated that more than 2,300 acetylcholine quanta may be released by a single nerve impulse under the conditions given in figure 1 (see Methods and Materials). This is approximately two orders of magnitude greater than the normal levels of acetylcholine release at the mouse neuromuscular junction.[12] In this regard, frequencies of stimulation higher than 1 Hz (such as those used for the TOF) under the conditions given in figures 1 and 2 produce an uncharacteristically steep rundown of acetylcholine release. As shown in figure 3A, when the TOF (fig. 3) stimulation parameters were applied under the experimental conditions given in figures 1 and 2 (*i.e.,* increased levels of acetylcholine secretion), measureable levels of acetylcholine release were eliminated after four stimuli at 2 Hz. Indeed, in all four preparations studied under these conditions, in contrast to the generally observed 30–50% reduction in twitch tension after four stimuli in TOF monitoring,[8] the EPPs declined to nonmeasureable levels after four to six stimuli at 2 Hz whereas Ca^{2+} currents were still detectable. The likely explanation for the results of figure 3A is that depletion of the immediately available store of acetylcholine occurs when frequencies of stimulation of 2 Hz or more are used under these conditions. In support of this hypothesis, a decrease in the population of acetylcholine-containing synaptic vesicles and elimination of EPPs may be observed with a single stimulus under similar conditions of K^+ channel blockade and increased Ca^{2+} concentrations.[11]

Depression of Ca²⁺ Currents and Neurotransmitter Release at Physiological Levels of Secretion

It has been found that Ca^{2+} currents may be accurately measured at more physiological levels of acetylcholine release at mouse neuromuscular junctions bathed in solutions containing reduced Ca^{2+} concentrations and lower concentrations of K^+ channel blockers (*physiological Ca^{2+} current solution*[15]). Such solutions also produce Ca^{2+} currents that are of brief duration and thus are more representative of the Ca^{2+} currents that mediate acetylcholine release. A series of experiments were made to determine whether the control level of quantal acetylcholine release in response to single nerve impulses is of the appropriate order of magnitude to resemble that seen at the murine and human neuromuscular junctions. The results of these experiments reveal that the average number of quanta of acetylcholine released by a nerve impulse under such basal conditions in this solution ranged from 21 to 298 quanta with a mean = 123 ± 38

quanta (n = 7 preparations). This level of release is similar to estimates of the normal levels of acetylcholine release at the human skeletal neuromuscular junction (~100 quanta[13]).

Figure 3B shows an experiment in which the average number of acetylcholine quanta released was 71 quanta, which is between the normal quantal output in the mouse (36 quanta[12]) and human[13] neuromuscular junctions. In the experiment described in figure 3B, when the TOF depression was measured in this solution, the pattern of rundown of EPPs with repetitive stimulation (upper traces) resembles that observed with published twitch tension measurements after block by nondepolarizing blockers such as tubocurarine, which, thus far, was used in these experiments to reduce EPPs below threshold for action potentials, or the clinically used agent vecuronium.[5–8] Specifically, figure 3B (upper traces) shows the averaged EPPs during the TOF after four consecutive repeats of the TOF protocol (each TOF was separated by 15-s interval in this experiment). Note that the level of release, rather than declining to nonmeasurable levels by the fourth stimulus as observed at the high levels of release (fig. 3A), stabilizes at 39% of the control level in this experiment (*i.e.,* the TOF ratio = 0.39). Indeed, there is no statistically significant difference between the averaged response to the third and fourth stimulus ($P = 0.69$). The average data for EPPs from all experiments in *physiological Ca^{2+} current solution* are presented in figure 4. Note that the average TOF ratio in all five experiments is 0.42 ± 0.05 ($P < 0.001$). In other experiments, 2 Hz stimulation was continued beyond the four used in TOF monitoring and no further reductions in EPPs were observed. Specifically, no differences were observed when the response to the fourth stimulus was compared with the response to the fifth stimulus in this solution (n = 5 experiments, $P = 0.55$) or when the response to the fourth stimulus was compared with the average of the responses to the next five stimuli ($P = 0.183$). Similar results were found in studies of contracting human muscle.[8]

When Ca^{2+} currents are measured simultaneously in this solution, significant decreases in the ratio of the averaged fourth current to the first Ca^{2+} current are observed. Thus in the experiment of figure 3B, the averaged Ca^{2+} current peak declined to 78% of the control level (fig. 3B; lower traces; $P = 0.03$; n = 4 stimuli). In all five experiments represented in figure 3B, the Ca^{2+} current peak at the level of the fourth stimulus declined to a mean of $76.4 \pm 4.2\%$ of the control level ($P = 0.004$).

The experiments presented thus far have been made in the presence of the nondepolarizing neuromuscular-blocking drug tubocurarine. This agent has been reported to have presynaptic blocking effects and it may be that the depression of EPPs under conditions of the TOF stimulation is not a normal occurrence.[20] One way to determine whether this is a naturally occurring phenomenon or one dependent on tubocurarine is to perform the experiments in the presence of vecuronium, a neuromuscular blocker in widespread

clinical use, which is reported to have minimal presynaptic effects.[20] In all five experiments with vecuronium (6 μM), the TOF ratio is the same as that observed in TC (0.42 ± 0.01 in vecuronium, 0.42 ± 0.05 in tubocurarine; $P = 0.89$). In two experiments using vecuronium as the neuromuscular-blocking drug, the Ca²⁺ current peak declined to 76 ± 0.04% of the control level ($P = 0.004$; n = 5 stimuli) and 73 ± 0.05% of the control level ($P = 0.04$; n = 4 stimuli) between the first and fourth stimulus during the TOF protocol. These results were similar to those found using tubocurarine as the neuromuscular blocker. Thus, in each of the seven experiments (5 with tubocurarine and 2 with vecuronium), neuromuscular depression during TOF stimulation was associated with a significant decline in the Ca²⁺ current peak. Specifically the Ca²⁺ current peak declined to 75.8 ± 3.3% of the control level and the EPP declined to 42.0 ± 1.0% of the control level between the first and fourth stimulus during the TOF protocol ($P < 0.001$ for both waveforms; n = 7 experiments with tubocurarine and vecuronium).

Depression of Neurotransmitter Release in the Absence of Neuromuscular-blocking Agents

Another more direct test of the physiological importance of TOF depression would be the measurement of neuromuscular depression in the absence of any blocking drugs in normal physiological salt solutions to determine whether neuromuscular depression occurs in the absence of nicotinic acetylcholine receptor blockade. Indeed, when the TOF decrement of muscle tension occurs in a clinical setting, the decline in twitch tension is due to individual muscle fibers in the entire muscle losing their action potentials and associated twitches, thus reducing the magnitude of the overall twitch of the muscle.

Performing electrophysiological experiments on contracting muscle is technically demanding and cannot be used to quantify acetylcholine secretion when all the EPPs are suprathreshold, as is the case at most end-plates. It was possible in four experiments to obtain impalements of the muscle in which EPPs and superimposed action potentials could be measured without dislodging the recording electrode and where the envelope of the EPPs that underlie the action potentials and subsequent subthreshold EPPs could be recorded. Figure 5A shows a representative experiment in which the level of quantal acetylcholine output was 21 quanta. This value reflects the low end of the range of quanta normally released by an action potential,[12] but it allows for accurate measurements of the TOF in the complete absence of blocking drugs in normal physiological salt solution. In this experiment, the first stimulus produced suprathreshold EPPs with an action potential (fig. 5A, AP), the second stimulus produced EPPs with a localized abortive action potential, and the two subsequent stimuli produced two progressively smaller subthreshold EPPs. The TOF was then repeated twice more (with a 15-s interval between TOFs), and the mean EPP component of the end-plate waveform decreased from an estimated value of 41.4 ± 1.5 mV with the first stimulus in the train to 18.2 ± 0.8 mV in response to the fourth stimulus (n = 3 repeats), producing a highly significant TOF ratio of 0.41 in this experiment ($P < 0.001$). The results from all four experiments are shown in figure 5B, with the mean TOF ratio being 0.49 ± 0.06 in the absence of neuromuscular-blocking drugs. There are no statistically significant differences between the TOF ratio in the absence of blocking drugs or in the presence of the nondepolarizing blockers tubocurarine or vecuronium ($P = 0.47$). The results of figure 5 thus confirm that the TOF is a real physiological phenomenon and not due to the presence of nondepolarizing neuromuscular-blocking drugs or a consequence of using a bathing solution of modified composition to make simultaneous measurements of Ca²⁺ currents and EPPs.

Discussion

The results of this study suggest that the mechanism underlying the depression of neutrally evoked muscle twitches, used as a diagnostic tool by clinical scientists during TOF monitoring, is more likely to be due to a decline in the nerve terminal Ca²⁺ current during brief 2 Hz stimulation than to depletion in the immediately available store of neurotransmitter. To address the issue of neuromuscular depression during TOF monitoring at normal levels of acetylcholine release, it was necessary to choose conditions in which both release and Ca²⁺ currents could be monitored and in which physiological levels of acetylcholine secretion were studied. *Physiological Ca²⁺ current solution* fulfils all of these criteria as this solution: (1) allows TOF depression to be studied at normal levels of release, (2) enables Ca²⁺ waveforms devoid of prolonged depolarization to be measured, and (3) precludes the presynaptic effects of tubocurarine. Depression of acetylcholine release during TOF stimulation in these solutions was associated with decreases in nerve terminal Ca²⁺ currents (fig. 3B) as it was at lower frequencies of nerve stimulation and higher levels of acetylcholine release (figs. 1 and 2). Indeed, although depletion of available quanta is an important limitation on the fidelity of synaptic transmission at central synapses[20–23] and at higher frequencies of stimulation and higher release rates at the neuromuscular junction (fig. 3A; studies by Christensen *et al.* and Silinsky[1,2]), the mechanism of rundown of twitches and the electrophysiological correlates of section (EPPs) can be fully explained by a decline in Ca²⁺ entry into the nerve ending.

The magnitude of prejunctional neuromuscular depression is controlled by nerve terminal receptors that include nicotinic receptors for the neurotransmitter itself[24,25] and receptors for important neuromodulators such as adenosine.[3] Thus, one potentially complicating issue in these studies is the need for neuromuscular-blocking drugs in most of these experiments, drugs that have been found to have

effects on the levels of neuromuscular depression.[24,25] For example, it has been shown that tubocurarine itself can enhance prejunctional depression by blocking prejunctional nicotinic receptors, receptors that normally exert a positive feedback effect on acetylcholine release.[24,25] The focus on depression during low-frequency stimulation in current study minimizes these concerns as the prejunctional effects of nicotinic receptor blockers only appear at higher stimulation frequencies.[24,25] In addition, the depressant effects of tubocurarine would not be a problem in the TOF experiments as the lower Ca^{2+} concentrations used for experiments given in figures 3B and 4 preclude the presynaptic effects of tubocurarine, even at higher stimulation frequencies.[24] Furthermore, similar results were found when experiments were performed in the presence of vecuronium, which under these conditions is believed to be devoid of presynaptic effects.[25] Finally, under conditions in which EPPs can be measured in contracting muscle in normal solutions in the absence of blocking drugs, the electrophysiological correlates of TOF depression can be observed (fig. 4). These results suggest that neuromuscular depression that occurs during TOF monitoring is likely to be caused by a decline in the nerve terminal Ca^{2+} currents and not by the presynaptic effects of nondepolarizing neuromuscular-blocking drugs. These results in no way minimize the importance of these prejunctional nerve terminal receptors in controlling the levels of neuromuscular depression at higher frequencies of nerve stimulation than those used here.

These current results are consistent with the finding that an important component of depression in response to nerve stimulation at the mammalian phrenic nerve involves a decline in the probability of release with repetitive stimulation.[1] As the probability of release is intimately related to the entry of Ca^{2+} through voltage-gated Ca^{2+} channels,[2,26] these results with Ca^{2+} currents provide a mechanistic handle on earlier reports that decreases in the immediately available store of acetylcholine would not fully explain neuromuscular depression.[1] These current results are also supported by experiments in the Calyx of Held in the rat brainstem,[26] in which inhibition of presynaptic P/Q-type channels (the same subtype present at mouse motor nerve endings)[15] is responsible for short-term depression during brief stimulation under a variety of stimulation conditions.[26] It should be noted that in current study, the rundown of acetylcholine release occurs at normal levels of neurotransmitter secretion in which nerve terminal Ca^{2+} currents can be measured (figs. 3 and 4) and in normal physiological solutions in the presence or absence of neuromuscular-blocking drugs (fig. 5). It thus seems that depression of acetylcholine secretion during low-frequency motor nerve stimulation is a real phenomenon inherent to the neuromuscular junction and not an artifact of the experimental conditions.

What causes the decline in Ca^{2+} currents during brief 2 Hz stimulation conditions used for TOF monitoring, and could the mechanism underlying this effect suggest a potential therapeutic target for neuromuscular disease? Indeed, similar frequencies of stimulation are used to test for neuromuscular diseases; therefore, elucidating that the mechanism of action of the decline in Ca^{2+} currents could prove useful for diagnostic and therapeutic purposes.[4,9] One possibility is that decline in Ca^{2+} currents is an intrinsic property of the P/Q type Ca^{2+} channel (e.g., Ca^{2+} channel inactivation). Another potential candidate is endogenous adenosine, which is derived from coreleased adenosine triphosphate,[3,27,28] and mediates depression of EPPs in frog[3] and mouse muscle[26,27] with continuous nerve stimulation. Although it is unknown whether enough endogenous adenosine derived from adenosine triphosphate can be generated within the 500-ms interpulse interval during TOF stimulation at normal levels of release at the mammalian neuromuscular junction, at higher levels of release and lower frequencies of stimulation (0.017 Hz) endogenous adenosine inhibits acetylcholine release via A_1 adenosine receptors at amphibian neuromuscular junctions.[3] In addition, endogenous adenosine inhibits acetylcholine release at both frog[3] and mammalian[26,27] neuromuscular junctions at normal levels of release. These results raise the possibility that selective adenosine A_1 adenosine receptor antagonists (theophylline and its derivatives) can be used therapeutically to assist in the treatment of neuromuscular disorders such as myasthenia gravis, in which the prejunctional depression can produce life-threatening effects.

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