Effect of Temperature on Endplate Potential Rundown and Recovery in Rat Diaphragm

MICHELLE MOYER AND ERIK VAN LUNTEREN
Pulmonary Division, Department of Medicine, Case Western Reserve University; and Louis Stokes Cleveland Department of Veterans Affairs Medical Center, Cleveland, Ohio 44106

Received 13 June 2000; accepted in final form 14 February 2001

INTRODUCTION

During continuous stimulation of the neuromuscular junction, there is an initial, rapid decline in end-plate potential (EPP) amplitude followed by an extended phase of slow decline in EPP amplitude (Bazzy 1994; Hubbard and Wilson 1973; Sosa and Zengel 1993; Wilson 1979; Zengel and Sosa 1994). In contrast, Hong and Chang (1989) examined the effect of envenomation on EPP amplitude rundown and observed only mild rundown at a warmer temperature of 37°C, which seemingly contradicted earlier studies.

One of the more commonly accepted hypotheses of EPP amplitude rundown and recovery is that the docked vesicle pool is depleted at the neuromuscular junction and the subsequent recovery is caused by replenishment of the depleted docked pool (Wu and Betz 1998; Zuckier 1989). Wu and Betz (1998) proposed a model in which synaptic vesicles reside in three pools: 1) a docked pool that is located at the membrane and is ready for immediate release; 2) a reserve pool that resides away from the membrane and replenishes the docked pool; and 3) a fused pool that consists of vesicles exposed to extracellular fluid during the time between exocytosis and endocytosis and that replenishes the reserve pool. In this model, the integrity of neurotransmission is maintained by the rate of replenishment of the depleted docked pool. Most cellular processes speed up at higher temperatures; accelerated rundown at increased temperatures might therefore be expected. In the proposed model of EPP amplitude rundown and recovery, however, there are two different steps that, if affected differentially by temperature, would lead to two different results when temperature is altered. Accelerated depletion of neurotransmitter at higher temperatures should lead to increased rundown whereas accelerated repletion of neurotransmitter at higher temperatures should lead to decreased rundown and/or increased recovery. The balance between these processes at physiological temperatures compared with cooler temperatures affects the ability of the neuromuscular junction to maintain neurotransmission. Vesicle recycling is known to be highly energy dependent (Palfrey and Artalejo 1998; von Gersdorff and Mathews 1999) and therefore would be expected to be quite temperature sensitive. Therefore, the present study...
tested the hypothesis that low temperature increases the rate of EPP amplitude rundown.

METHODS

Male Sprague-Dawley rats (250–350 g) were anesthetized with urethane (initial dose 1g/kg i.p. with additional doses of 0.1–0.2 g/kg i.p. given as needed). All experiments were performed in accordance with the animal care and welfare guidelines of the National Institutes of Health. The diaphragm and both phrenic nerves were removed surgically. The muscle, left intact to the ribs and the central tendon, was stretched out and pinned in a Sylgard-lined 35-mm petri dish. Oxygenated Krebs solution composed of (in mM) 135 NaCl, 5 KCl, 2.5 CaCl₂, 1 MgSO₄, 1 NaH₂PO₄, 15 NaHCO₃, and 11 glucose, pH adjusted to 7.25–7.35, and bubbled continuously with 95% O₂-5% CO₂ flowed into a petri dish to superfuse the phrenic nerve hemidiaphragm preparation. Overflow was evacuated by suction. To minimize vibration during electrophysiological recording, the solution was not bubbled directly in the petri dish. A dissolved oxygen meter (World Precision Instruments, Sarasota, FL) was used to verify that this set-up was well-oxygenated. Temperature was regulated in the petri dish with a Peltier device (Medical Systems, Greenvale, NY) and monitored with a thermostor.

Intracellular membrane potentials and EPPs of muscle fibers were recorded using glass microelectrodes fabricated with a Flaming Brown micropipette puller (Sutter Instruments, Novato, CA) (resistance 5–15 MΩ when filled with 3 M KCl). Microelectrodes were lowered slowly by a micropositioner (David Kopf Instruments, Tujunga, CA) in the region of the end-plate, which was indicated by the presence of miniature end-plate potentials (MEPPs). Muscle action potentials were inhibited after 15 minutes of equilibration with conotoxin (Bachem, King of Prussia, PA) (Bazzy 1994; Breugelmans and Bazzy 1997; Hong and Chang 1989, 1991; Moyer and van Lunteren 1999), which preferentially blocks muscle over nerve sodium channels in concentrations of 2.5–4 μM (Cruz et al. 1985; Prior et al. 1993). All chemicals other than conotoxin were obtained from Sigma Chemical (St. Louis, MO).

EPPs were evoked by supramaximal stimuli applied to the phrenic nerve (pulse width 0.2 ms) via a suction electrode (A-M Systems, Everett, WA). Muscle fibers underwent stimulation at a frequency of 20 or 50 Hz for a minimum of 30 s or 600 pulses. The frequencies chosen are in the range of phrenic and limb muscle motor unit firing frequencies reported in the rat (Hennig and Lomo 1985; Kong and Berger 1986). During stimulation, trains of pulses with a train duration of 0.33 s were delivered once per second, thereby allowing the neuromuscular junction a 0.67 s recovery period before the onset of the subsequent train. Thus each train consisted of seven pulses for 20-Hz stimulation and 17 pulses for 50-Hz stimulation. Sample sizes were 10 hemi-diaphragms for each experimental arm. Each hemi-diaphragm underwent only a single stimulation paradigm; thus a total of 40 hemi-diaphragms were used in this study. Potentials were recorded with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA), digitized, collected on-line (Axotape software, Axon Instruments), and stored on the hard drive of a computer for future analysis.

EPPs were analyzed with manually controlled cursors that measured the peak height of each potential as a function both of time and of pulse number, the latter to correct for the variations in pulse number per unit of time that occurred during the different stimulation protocols. To compare rate of EPP rundown among the various stimulation paradigms, each EPP was normalized to the amplitude of the first EPP in the stimulation protocol. Cumulative EPP amplitude curves were calculated from the normalized EPP amplitudes added together consecutively. All values are means ± SE. Statistical analysis of EPP amplitude in each train, and of changes in the first, seventh, and fourteenth and 17th EPPs over time, was done with repeated measures two-way ANOVA, which was followed by Student’s Newman Keuls test when ANOVA indicated statistical significance. Cumulative EPP amplitudes were analyzed with an unpaired t-test to test for differences among temperatures. For statistical analysis, a P value of <0.05 (two-tailed) indicated significance.

RESULTS

The mean resting membrane potential at the onset of stimulation was −72.6 ± 0.7 mV. The mean EPP size at the onset of stimulation was 23.7 ± 0.9 mV. Initial mean membrane potential and initial mean EPP size did not differ significantly among treatments. However, there was a trend toward higher EPP sizes at warm temperatures for the 20 Hz stimulation data (see legends for Figs. 2 and 3).

Figure 1 shows examples of EPPs recorded at 20 and 37°C during 50-Hz stimulation. The EPP amplitudes ran down more within both the initial and the tenth trains during stimulation at 20°C than they did during stimulation at 37°C. Mean values for EPP amplitude rundown during 50-Hz intermittent stimulation are depicted as a function of time and pulse number in Fig. 2. There was a clear difference in intra-train EPP amplitude rundown at 20°C compared with 37°C, which was seen as early as the first train during 50-Hz stimulation. This was statistically significant by the third pulse of the first train and continued to be significant during trains 2, 3, 4, and 5. At 20°C, there was rapid rundown through a great portion of the train whereas at 37°C the EPP amplitude within the trains decreased rapidly until peaks 5 and 6, after which there was a plateau with very little additional EPP rundown. Even by train 5, the plateau was still evident toward the end of the train at both temperatures.

The results of EPP amplitude rundown during 20-Hz intermittent stimulation tended to be milder than those at 50-Hz stimulation (Fig. 3). There was gradual rundown within each train at each temperature. Intra-train rundown tended to be greater at 20°C than at 37°C so that by the sixth pulse of the third train there was a significant difference between the EPP amplitudes at each temperature, with the EPP amplitude being lower at 20°C. These differences continued to be significant during trains 4 and 5.

The maintenance of EPP amplitude as a function of stimulation frequency during the first, seventh, and fourteenth
pulses in each train is illustrated in Fig. 4 so that EPP rundown over a longer time period could be examined. (The seventh pulse was the last EPP in a 20-Hz train and the seventeenth pulse was the last EPP in a 50-Hz train.) During a period of 30 s, the first EPP in the train was significantly lower at 20°C than it was at 37°C during stimulation both at 20 and at 50 Hz. At 20 Hz, after 10 s of stimulation, significant differences at each temperature were seen between the first and seventh EPP amplitudes in the train. At 50-Hz stimulation, significant differences between the first and seventh EPP amplitudes were also seen at each temperature. At 20 Hz, significant differences at each temperature were seen between the first and seventh EPP amplitudes in the train. At 50-Hz stimulation, significant differences between the first and seventh EPP amplitudes were also seen at each temperature.

Cumulative EPP amplitude values were calculated by successively adding the amplitudes of each EPP during the stimulation period; this provided an estimate of total neurotransmitter release in the neuromuscular junction (Fig. 5). EPPs were normalized relative to EPP amplitude at the onset of stimulation, which was given a value of 1 unit. Normalization was done to eliminate the effects of small variability in absolute EPP size (in mV) among the neuromuscular junctions because a slight difference in absolute EPP amplitude at the onset of stimulation would eventually have a large cumulative effect. During 20-Hz stimulation, the cumulative EPP amplitude was significantly lower at 20 than at 37°C, both by pulse 300 and by pulse 600 ($P < 0.001$ and $P < 0.002$, respectively). During 50-Hz stimulation, the cumulative EPP amplitude was significantly lower at 20 than at 37°C, both by pulse 300 and by pulse 600 ($P < 0.000001$).

**DISCUSSION**

During intermittent stimulation, EPP amplitude rundown was greater at cold temperatures than it was at warm temperatures. Intra-train rundown was dependent on temperature during the initial period of stimulation and persisted throughout the stimulation period, with the decrement being more extreme at 20 than at 37°C. The difference was significant as early as the first train during 50-Hz stimulation and the third train during 20-Hz stimulation. In contrast, there were minimal effects of temperature on inter-train rundown during the early trains. The temperature effects of inter-train rundown were seen by 10 s of stimulation at both frequencies, with inter-train rundown also being greater at cool temperatures than at warm temperatures.

There are many proposed theories of EPP amplitude rundown and it is interesting to examine these mechanisms in the context of the data from the present study. Several investigators suggested that endogenously released adenosine is a modulator in neuromuscular depression (Meriney and Grinnell 1991; Redman and Silinsky 1994). ATP is co-released with acetylcholine when the neuromuscular junction is stimulated (Silinsky 1975; Smith 1991). ATP is then hydrolyzed to its...
metabolite, adenosine, which has a presynaptic inhibitory effect (Ginsborg and Hirst 1972; Ribeiro and Sebastiao 1987; Silinsky 1980, 1984). The possible inhibitory mechanism is currently unknown but could be caused by a reduction in Ca$^{2+}$ entry (Hamilton and Smith 1991) or a reduction in acetylcholine release caused by Ca$^{2+}$ (Silinsky 1981, 1984; Silinsky and Solsona 1992). The effect of temperature on ATP release has not been studied but, at physiological temperatures, ATP has been shown to be released in stoichiometric amounts, with acetylcholine, from the motor nerve endings (Schweitzer 1987; Silinsky 1975; Silinsky and Redman 1996; Smith 1991; Smith and Lu 1991). Because the hydrolysis of ATP to adenosine is a temperature-dependent process (Palfrey and Artalejo 1998; von Gersdorff and Mathews 1999), a low temperature should slow the hydrolysis of ATP following its release after stimulation. Thus, according to this model, less adenosine would produce less neuromuscular junction depression. The results of the present experiment found the opposite: that there was, in fact, more depression at a low temperature. This suggests that, in the rat neuromuscular junction after 20- and 50-Hz intermittent stimulation, the ATP metabolite adenosine was not the major factor accounting for the effects of low temperature on the depression of EPP amplitude. However, more investigation is required to rule out the possibility that, under cold temperatures, other energy-requiring processes might consume less ATP, which would result in the release of more ATP and, therefore, more adenosine generated in the synaptic cleft. If this were the case, the increased adenosine production could cause greater depression in cold temperatures.

Other investigators also found evidence of fast endocytosis (Hsu and Jackson 1996; Neves and Lagnado 1999). This rapid process, called “kiss and run,” involves vesicles briefly fusing
with the plasma membrane and releasing neurotransmitter through a fusion pore (Cousin and Robinson 2000). The vesicle then immediately pinches off without ever integrating with the plasma membrane. Still others found evidence that this rapid process does not play a major role in vesicle recycling (Cousin and Nicholls 1997; Reuter and Porzig 1995; Ryan and Smith 1995; Wu and Betz 1998). The fastest time constants reported for rapid endocytosis were \( \sim 440 \) ms (Hsu and Jackson 1996), which is in contrast with the data of Reuter and Porzig (1995), Ryan and Smith (1995), and Wu and Betz (1998), who reported that the fastest time constants of vesicle recycling are on the order of \( 10-20 \) s. Wu and Betz (1998) measured mobilization from reserve to docked pools and found a time constant (\( \tau \)) of \( \sim 12 \) s after \( 10 \) s of stimulation. They found that \( \tau \) depended on stimulus duration and, when the duration was increased from \( 10 \) to \( 300 \) s, that \( \tau \) increased by a factor of six. They did not measure \( \tau \) for stimulation periods as short as \( 333 \) ms, as in the present study, however. It is likely that, because there was very little inter-train rundown between stimulus trains evoked every second, the results of the present study follow the trend of the data from Wu and Betz (1998). However, more experiments are needed to fully describe the time constant of recovery from EPP amplitude depression. A more recent study (Richards et al. 2000) used the differential staining properties of fluorescent dyes to elucidate two different recycling routes that fill two vesicle pools in the frog neuromuscular junction. They found a slow pathway that passes through cisternal intermediates to fill the reserve pool and a small-capacity fast pathway that fills the readily releasable pool directly and is promoted by high \([Ca^{2+}]_i\). This model, which could also explain the present data, has elements of the fast endocytosis model and the Wu and Betz (1998) model (see following paragraph). The fast direct refilling of the immediately releasable pool may be slowed by low temperature, thus causing an increase in EPP amplitude rundown. Clearly, further investigation is needed to more fully define the role of fast endocytosis in vesicle recycling. However, a majority of the data obtained thus far suggests that this process is probably not significantly involved in intra-train recovery but that it may play a role in inter-train recovery during trains as short as \( 333 \) ms.

Many investigators have suggested that depression of EPP amplitude at the neuromuscular junction is caused by a decrease in neurotransmitter release (Wilson 1979; Wu and Betz 1998; Zucker 1989). A recent model involving replenishment by mobilization from a larger reserve vesicle pool to a depleted readily releasable pool (Wu and Betz 1998; Zucker 1989) can explain the present findings. Because, as stated in this model, EPP amplitude depression is caused by the depletion of the readily releasable docked pool, subsequent recovery of EPP amplitude depends on the mobilization of reserve neurotransmitter vesicles to replenish the docked pool. However, the present study did not directly measure neurotransmitter recycling and therefore cannot differentiate between other neurotransmitter replenishment mechanisms discussed previously (see preceding paragraph). If the present data do indeed reflect the recycling and recovery model of Wu and Betz (1998), it is possible that, in the present study, the fast intra-train decline and the near-complete recovery of EPP size by the onset of the subsequent train reflect the depletion and replenishment of the docked pool and that the slower inter-train decline in EPP size reflects depletion of the reserve pool. The greater decline in EPP amplitude during stimulation at \( 20^\circ C \) could be a result of the decrease in mobility during cool temperatures, which would mean that the docked pool is replenished from the reserve pool more slowly at a temperature of \( 20^\circ C \) than at a temperature of \( 37^\circ C \). Because inter-train rundown is proposed to be caused by the depletion of the reserve pool and to depend less on mobilization, this would result in a less temperature-dependent process. Thus inter-train rundown would be affected only modestly by temperature. The temperature dependence of recycling is supported by the data of David and Barrett (2000), who showed that mitochondrial \( Ca^{2+} \) sequestration was reduced in the mouse neuromuscular junction at cool temperatures. These authors speculated that the temperature dependence could be caused either by a reduction in \( Ca^{2+} \) uptake via mitochondrial uniporters or by an increase in \( Ca^{2+} \) extrusion via exchangers.

One of the interesting results of the present study was that, following the initial rapid decline in EPP amplitude, a plateau occurred throughout the remainder of the train at \( 50 \) Hz at \( 37^\circ C \) but not at \( 20^\circ C \). This suggests that, at the higher temperature, there was enough replenishment of the docked pool to maintain EPP amplitude at a constant level after the initial decline, even with maintained stimulation. At the cooler temperature, however, there was not enough replenishment of the docked pool and, therefore, EPP amplitude declined after prolonged stimulation. EPP size for neuromuscular junctions stimulated at \( 20 \) Hz tended to decrease throughout each train, but the reductions were modest. This could be explained by the theory that the more moderate activation at \( 20 \) Hz did not fully deplete the docked neurotransmitter pool supply so that mobilization was not the determining factor in determining EPP amplitude rundown.

Two different stimulation frequencies were used in the present study so that frequencies in the phrenic and limb muscle physiological firing ranges would be included. In the rat, phrenic motoneurons have discharge rates between \( 34 \) and \( 76 \) Hz, with a mean of \( 56 \) Hz during eupnea (Kong and Berger 1986). Also, motor units of limb muscles were found to have firing frequencies between \( 18 \) and \( 91 \) Hz (Hennig and Lomo 1985).

There was a larger initial mean EPP for the \( 20 \) Hz, \( 37^\circ C \) data because a few fibers had slightly larger initial EPP amplitudes. We investigated whether this could have skewed our results. When the three highest-amplitude samples were eliminated, the mean initial EPP of the remaining fibers was not significantly different from the mean initial EPP at \( 20^\circ C \). We retested the EPP amplitude rundown and found that the data were not affected by the elimination of these three samples. The \( 50 \) Hz initial EPP amplitude data did not differ between \( 20 \) and \( 37^\circ C \). Thus, any differences in initial EPP size are unlikely to explain why temperature effects EPP rundown.

In conclusion, the results of the present study indicate that the rundown and subsequent recovery of EPP amplitude during intermittent stimulation is greater at \( 20^\circ C \) than it is at \( 37^\circ C \). It is thought that the process of rundown is caused by a depletion of the readily releasable neurotransmitter pool and that the subsequent recovery is caused by the replenishment of that pool. The present study does not directly measure the process of neurotransmitter recycling and therefore does not exclude the possibility that our data support other models for EPP
amplitude rundown and subsequent recovery. According to the most widely accepted model of recycling (Wu and Betz 1998), the preservation of EPP amplitude caused by mobilization of neurotransmitter is thought to be a key factor in preserving neuromuscular junction function. The present data are consistent with this model as well as with other possible models. The data from the present study show a significant difference in EPP amplitude rundown between cold and warm temperature conditions during 20- and 50-Hz stimulation; this suggests that the vesicle replenishment process has high activation energy that is affected considerately by temperature.

This study was supported in part by the Medical Research Service of the Department of Veterans Affairs and by a Specialized Center of Research grant from the National Heart, Lung, and Blood Institute (HL-42215).

REFERENCES

HSU S-F AND JACkSON MB. Rapid exocytosis and endocytosis in nerve terminals of the rat posterior pituitary. J Physiol (Lond) 494: 539–553, 1996.
SILINSKY EM. On the mechanism by which adenosine receptor activation inhibits the release of acetylcholine from motor nerve endings. J Physiol (Lond) 346: 243–256, 1984.