Effects of DAP on diaphragm force and fatigue, including fatigue due to neurotransmission failure

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Van Lunteren, Erik, and Michelle Moyer. Effects of DAP on diaphragm force and fatigue, including fatigue due to neurotransmission failure. J. Appl. Physiol. 81(5): 2214–2220, 1996.—Among the aminopyridines, 3,4-diaminopyridine (DAP) is a more effective K+ channel blocker than is 4-aminopyridine (4-AP), and, furthermore, DAP enhances neuromuscular transmission. Because 4-AP improves muscle contractility, we hypothesized that DAP would also increase force and, in addition, ameliorate fatigue and improve the neurotransmission failure component of fatigue. Rat diaphragm strips were studied in vitro (37°C). In field-stimulated muscle, 0.3 mM DAP significantly increased diaphragm twitch force, prolonged contraction time, and shifted the force-frequency relationship to the left without altering peak twitch force, tetanic force, resulting in increased force at stimulation frequencies ≤50 Hz. During 20-Hz intermittent stimulation, DAP increased diaphragm peak force compared with control during a 150-s fatigue run and, furthermore, significantly improved maintenance of intratrain force. The relative contribution of neurotransmission failure to fatigue was estimated by comparing the force generated by phrenic nerve-stimulated muscles with that generated by curare-treated field-stimulated muscles. DAP significantly increased force in nerve-stimulated muscles and, in addition, reduced the neurotransmission failure contribution to diaphragm fatigue. Thus DAP increases muscle force at low-to-intermediate stimulation frequencies, improves overall force and intratrain fatigue during 20-Hz intermittent stimulation, and reduces neurotransmission failure.

Methods

Studies were performed in vitro on diaphragm muscle of 250- to 350-g Sprague-Dawley rats. The animals were anesthetized with urethan (1 g/kg), and the diaphragm was removed surgically. For several studies the phrenic nerves were also dissected out and removed intact with the diaphragm. The muscles were placed in oxygenated (95% O2-5% CO2) physiological solution consisting of (in mM) 135 NaCl, 5 KCl, 2.5 CaCl2, 1 MgSO4, 1 NaH2PO4, 15 NaHCO3, and 11 glucose, with the pH adjusted to 7.3–7.4. Small strips (1–1.5 mm diameter) were made, with care taken to keep the rib origin and central tendinous insertion intact. For those studies in which the phrenic nerve was also removed, identically sliced strips were made, each containing the nerve connected to the diaphragm. The muscles were mounted vertically in a double-jacketed bath (37°C) containing physiological solution. Except as noted below, the muscles were field stimulated electrically (1-ms pulse width, supramaximal voltage) at optimal length via platinum electrodes, and force was measured with a high-sensitivity isometric transducer (Kent Scientific/Radnotti Glass Technology, Monrovia, CA). This method routinely produces diaphragm twitch tensions of ~0.5 kg/cm2 (30). With this stimulation paradigm, addition of curare to the bath does not reduce muscle force, indicating that the muscles are activated directly as opposed to via phrenic nerve branches. A small group of muscle strips underwent stimulation via the phrenic nerve by means of a suction electrode (A-M Systems, Everett, WA) with a pulse width of 0.2 ms and supramaximal voltage.

Diaphragm muscle strips underwent isometric twitch stimulation at a frequency of 0.1 Hz to establish a baseline for twitch force. Muscle strips in which force varied by >5% during the predrug baseline period were rejected from further analysis. After the 3-min baseline period, aliquots of DAP...
dissolved in physiological solution were added to the bath, and twitch stimulation at 0.1 Hz was continued for 10 min so that the muscle twitch force had stabilized after drug addition. Additional physiological solution was added to control (no drug) strips. For the muscle field-stimulated arm of the nerve vs. the muscle field-stimulated study, d-tubocurare (12 µM) was added to the bath solution before stimulation to ensure that direct stimulation of intramuscular nerve branches was entirely absent. With the exception of muscle strips used to assess dose-response and force-frequency relationships, all muscles then underwent stimulation with 20-Hz trains. The train duration was 330 ms, and trains occurred every second. A stimulation frequency of 20 Hz was chosen, because diaphragm motor units normally fire at a frequency of 10–30 Hz during breathing (26). In all protocols, n ≥ 5. Reagents and drugs were obtained from Sigma Chemical (St. Louis, MO).

Muscle force records were digitized, collected on-line (Axo-tape, Axon Instruments, Foster City, CA), and stored on the hard drive of a computer. On-screen measurements of force were made with manually controlled cursors. Isometric tension was measured in grams. Because of interstrip variability in size, force was normalized relative to the last three twitches during the baseline period. Isometric twitch kinetics were quantified by measurements of the time to peak force (contraction time) and the time for peak force to decay by 50% (half-relaxation time) with use of single twitches or the first and last twitch, respectively, of the tetanus. During fatigue, the plateau phase of a tetanic contraction, normally maintained at a constant force, decreases over time during each 20-Hz train (2, 27). To measure this degree of intratetanic fatigue, the force at the end of the 330-ms-long train was measured and expressed as a percentage of the maximum tetanic force within the same tetanus (force-330) (27).

The NF contribution to diaphragm fatigue was calculated by comparing force loss during repetitive nerve stimulation with that during repetitive muscle field stimulation with use of procedure 2 of Kuei et al. (15). The force decline during muscle field stimulation was due to muscle failure (MF), whereas the force decline in nerve-stimulated muscles (F) was due to the combined contribution of MF and NF. The formula was developed by Aldrich et al. (1) and used by Kuei et al.: NF = (F - MF)/(1 - MF), where F and MF were calculated by the percentage of force loss at each time point during nerve and muscle field stimulation, respectively.

Statistical analysis of the dose-response effects of DAP on prefatigue force and isometric twitch kinetics was performed with one-way analysis of variance followed by Dunnett’s test. Statistical assessment of the effects of DAP on the force-frequency relationship and on force and twitch kinetics during fatigue runs was performed with two-way repeated-measures analysis of variance followed by the Newman-Keuls test. P < 0.05 (2-tailed) was considered to indicate statistical significance.

RESULTS

Field-stimulated muscle DAP at 0.1–10 mM increased diaphragm twitch force, with the increase being greatest at 0.3–3.0 mM (Fig. 1, Table 1). At 0.1–3 mM DAP, force gradually increased over 5–10 min and was maintained at a plateau or near plateau thereafter for the remainder of the 15-min period after drug addition. In contrast, with 10 mM DAP, force reached a peak 3–4 min after drug addition and subsequently declined, reaching control values at 10 min and reaching a nadir of slightly less than one-half of control values at 15 min after drug addition. Contraction time was prolonged significantly by 0.3–10 mM DAP, whereas half-relaxation time was not significantly different from control values (Table 1). On the basis of these responses, further studies were performed with 0.3 mM DAP, inasmuch as this was the lowest concentration associated with a near-maximal increase in twitch force.

Figure 1 depicts effects of 0.3 mM DAP on the diaphragm force-frequency relationship. DAP shifted the relationship to the left, without altering peak tetanic force. Force was augmented significantly by DAP at stimulation frequencies =50 Hz, with the relative increase in force reaching a maximum of ~150% at a stimulation frequency of 10 Hz.

During long-term 20-Hz intermittent stimulation, in the absence of DAP, force progressively declined after an initial modest increase, whereas with DAP there was a larger initial potentiation of force before a decline in force (Fig. 3A). The effects of the DAP-induced force increase outweighed the subsequently more rapid rate of force decline over time, so that DAP increased diaphragm force for the fatigue run as a whole (P < 0.001). Force-330, an evaluation of the ability of the muscle to maintain force during the plateau phase within the same tetanic stimulation, was improved by DAP during 20-Hz stimulation (P = 0.0016; Fig. 3B). Peak force was elevated significantly by DAP for the first half of the fatigue run (Fig. 3A), whereas force-330 was elevated significantly by DAP during the second half of the fatigue run (Fig. 3B).

During repetitive 20-Hz stimulation the contraction time was significantly greater with than without DAP throughout the entire time frame (P = 0.0003; Fig. 4A); the extent of contraction time prolongation by DAP increased modestly over time. The half-relaxation time was also prolonged significantly by DAP (P < 0.0001; Fig. 4B), an effect that became considerably more pronounced over time. The substantially augmented
prolongation of relaxation time by DAP during fatigue and the consequential enhanced temporal summation of force (Fig. 1) likely contributed to the better maintenance of peak force in DAP-treated muscle strips during the course of the fatigue run.

Phrenic nerve-stimulated muscle. DAP significantly increased force in nerve-stimulated muscle strips during 20-Hz stimulation (P = 0.007; Fig. 5A) and in a manner that was qualitatively similar to that seen with field-stimulated muscle (cf. Figs. 3A and 5A). Rate of force decline and effects of DAP on field-stimulated muscle strips were similar in curarized and noncurarized preparations (data not shown). During 20-Hz intermittent stimulation the NF contribution to diaphragm fatigue rose to 45% in the absence of DAP (Fig. 5B). With the addition of DAP, NF was considerably less, reaching a plateau of ~20%.

DISCUSSION

The results of the present study indicate that DAP is a more effective skeletal muscle inotropic agent than 4-AP (30, 32), causing a greater increase in muscle force without accentuating fatigue during 20-Hz stimulation. Not previously described for the aminopyridines is the fact that they also reduce the degree of intratrain fatigue and attenuate the neuromuscular component of fatigue.

Previous investigators have demonstrated the greater K⁺ channel-blocking potency of DAP than 4-AP. For example, Kirsch and Narahashi (14) measured K⁺ channel conductance in squid giant axons and found that 1 mM 4-AP causes a 75% K⁺ current block, whereas 0.025 mM DAP produces the same amount of K⁺ current block. These authors suggested that this greater affinity may be due to the greater hydrogen-bonding ability of DAP. Molgo et al. (23) measured stimulus-evoked transmitter release recorded as end-plate potentials in single fibers of isolated mouse phrenic nerve-hemidiaphragm preparations. They found that DAP was six to seven times more potent than 4-AP in this respect. Our results are consistent with these findings, in that DAP augmented force to a greater extent than 4-AP during low-to-intermediate stimulation frequencies (Fig. 6A). DAP increased force an average of 137% over control values at stimulation frequencies ranging from 1 to 30 Hz, whereas previous experiments showed that 4-AP only increased force an average of 59% over this same stimulation frequency range (32). In preliminary studies we found that an increase in 4-AP concentration up to 2 mM did not augment diaphragm force any more than 0.3 mM 4-AP, suggesting that the greater increase in force with DAP than with 4-AP is not due to a shifted dose-response relationship.

DAP shifted the normal force-frequency response curve to the left. DAP prolongs action potentials as a consequence of a decreased rate of repolarization (6), thereby also prolonging contraction time. At a low-to-intermediate stimulation frequency, e.g., 20 Hz, prolongation of contraction time results in a higher degree of twitch fusion, causing an increase in force output. However, fusion is complete at high stimulation frequencies, e.g., 120 Hz, so that no additional force can be generated solely by prolonging contraction time.

The effects of DAP on muscle fatigue during 20-Hz field stimulation were qualitatively similar to previously reported effects of 4-AP (30), in that both agents increased force at the onset of stimulation, augmented the degree of force potentiation during the early part of repetitive stimulation, and improved force over time for the entire period of repetitive stimulation. A quantitative comparison of the effects of these two agents is depicted in Fig. 6B, which indicates similar quantitative effects of the two agents on muscle fatigue. During

### Table 1. Effects of 0.1-10 mM DAP on diaphragm isometric twitch force and twitch kinetics

<table>
<thead>
<tr>
<th>DAP, mM</th>
<th>No Drug</th>
<th>0.1</th>
<th>0.3</th>
<th>1.0</th>
<th>3.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Force, N</td>
<td>0.80 ± 0.17</td>
<td>0.56 ± 0.13</td>
<td>0.66 ± 0.51</td>
<td>1.97 ± 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction time, ms</td>
<td>25.5 ± 1.0</td>
<td>24.5 ± 1.7</td>
<td>29.0 ± 0.6</td>
<td>31.0 ± 1.7</td>
<td>28.0 ± 0.8</td>
<td>29.5 ± 0.5</td>
</tr>
<tr>
<td>Half-relaxation time, ms</td>
<td>21.0 ± 1.1</td>
<td>24.5 ± 2.5</td>
<td>23.0 ± 3.4</td>
<td>22.5 ± 2.6</td>
<td>20.0 ± 1.7</td>
<td>24.0 ± 1.5</td>
</tr>
</tbody>
</table>

Value are means ± SE. DAP, 3,4-diaminopyridine. Data for force are maximum increases over 15 min after drug addition. Contraction time and half-relaxation time are at time of maximum force increase. *Statistically significant differences between no drug and different drug concentrations (P < 0.05).
In the course of fatigue, there was an especially prominent prolongation of relaxation time in the DAP-treated muscles (Fig. 4B); this prolongation of relaxation time was similar to that observed previously for 4-AP-treated muscles (30) and results in considerable temporal summation of force (Fig. 1), leading to a better preservation of peak force.

Force-330 is an estimate of the muscle’s ability to maintain force during a short tetanic contraction. This measure of intraintrain fatigue is believed to reflect high-frequency fatigue, as opposed to serial measurements of peak force over time (intertrain fatigue), which is believed to reflect low-frequency fatigue (27). At 20-Hz stimulation, force-330 was better preserved over time in the presence than in the absence of DAP, suggesting that it is possible to partially ameliorate high-frequency fatigue with the aminopyridines. Reaud and Comtois (27) recently found that elevating extracellular K⁺ concentration further inhibited the maintenance of force during the plateau phase of a tetanic contraction in previously fatigued muscle, which is consistent with the present findings that preventing K⁺ efflux can reduce intratrain fatigue.

Two major categories of peripheral fatigue are contractile failure and transmission failure (3). Aldrich et al. (1) demonstrated a significant contribution of NF to muscle fatigue after high-frequency stimulation of isolated rat diaphragm, and Kuei et al. (15) noted that the relative contribution of NF to rat diaphragm fatigue increased progressively with higher rates of stimulation. Kuei et al. used two methods to estimate the neurotransmission component of fatigue: in procedure 1 they stimulated the phrenic nerve to produce fatigue and intermittently superimposed direct muscle stimulation, whereas in procedure 2 they compared separate groups of muscle strips that underwent fatiguing stimulation via field stimulation or via phrenic nerve stimulation (similar to the present study). A potential disadvantage of procedure 2 is that the field-stimulated muscle strips may maintain higher tensions than the nerve-

Fig. 3. A: effects of 0.3 mM 3,4-diaminopyridine on diaphragm force during intermittent stimulation at a frequency of 20 Hz. Force in all cases is normalized to value for twitch force immediately before addition of drug or no drug. B: effects of 0.3 mM 3,4-diaminopyridine on diaphragm's ability to maintain force during plateau phase within the same tetanic stimulation (F-330). F-330 was determined by evaluating force of last contraction in train as a percentage of maximum tetanic force of the same tetanic contraction at each time point. ○, No drug; ■, 3,4-diaminopyridine. Values are means ± SE. *Statistically significant differences between drug and no drug (P < 0.05).

Fig. 4. Effects of 0.3 mM 3,4-diaminopyridine on isometric contraction time (A) and isometric half-relaxation time (B) of diaphragm. ○, No drug; ■, 3,4-diaminopyridine. Values are means ± SE. *Statistically significant differences between drug and no drug (P < 0.05).
stimulated muscles as fatigue develops and, hence, may be more susceptible to a contractile type of fatigue. This effect would reduce the differences between the tensions elicited by direct and indirect stimulation and could lead to an underestimate of transmission failure. However, Kuei et al. found slightly higher estimates for the maximal relative contribution of NF to fatigue during procedure 2 than during procedure 1, arguing that this issue is not a major concern. Furthermore, in the present study, the same technique was used for the DAP-treated and untreated muscles, so that any systematic effects of the technique on quantification of the neurotransmission component of fatigue should apply equally to DAP-treated and untreated muscles.

DAP has been found to enhance synaptic transmission at the neuromuscular junction (5, 9, 10, 12, 13, 17, 18, 22, 23, 28). With DAP we found that the NF contribution to neuromuscular fatigue was decreased at a stimulation frequency of 20 Hz. It is believed that DAP causes an increased amount of neurotransmitter release. Katz and Miledi (12) measured end-plate potentials in frog muscle fiber to show that the amount of quantal neurotransmitter packets released per nerve impulse was several thousand with the addition of DAP compared with an average of only 300 without the drug. Also, electron-microscopic studies of motor nerve terminals show a large increase in the number of synaptic vesicles at the release site in the frog neuromuscular junction when an aminopyridine is present compared with no aminopyridine (11). The mechanisms of increased neurotransmitter release are still under debate; however, several common possibilities have been proposed on the basis of the belief that the neurotransmitter increase is due to an increased calcium flux at presynaptic nerve terminals (20). This may occur by the blockade of K⁺ channels (22), by an increase in the voltage-dependent calcium conductance in the nerve terminals (19), or by prolongation of the action poten-
tial at the nerve terminal (18, 22). The failure of the aminopyridines to enhance neurotransmitter release without calcium indicates that calcium flux at presynaptic terminals does play a key role in the increased amount of neurotransmitter released when DAP is present (18, 22).

The present study was performed in vitro, so effects of DAP may differ in vivo from those found here. For example, in vivo muscle fibers contract asynchronously in response to phrenic nerve traffic, whereas in vitro they contract synchronously in response to electrical stimulation. Furthermore, in vivo blood flow is intact and can be modulated in response to the metabolic demands of the muscle. These factors may have important influences on the muscle, especially during fatigue. On the other hand, the in vitro preparation has been used extensively for studies of muscle contractility and fatigue (1, 2, 6, 15, 16, 30–32) and has the advantage of isolating the muscle tissue from potentially confounding systemic influences. Thus it is possible that in vivo DAP may influence the pattern of phrenic motoneuronal recruitment and/or diaphragm blood flow and, hence, affect diaphragm performance indirectly. The present study was designed to assess the direct effects of DAP on the muscle and the neuromuscular junction, which was the rationale for the in vitro approach.

In conclusion, the present data indicate that DAP improves diaphragm contractile performance to an extent that is at least as great as that previously reported for 4-AP (16, 30, 32). In addition, we found that DAP improves intratrain fatigue and reduces the NF contribution to fatigue. Inasmuch as DAP has been associated with fewer adverse effects than 4-AP when administered to humans, DAP appears to be a better agent than 4-AP for future in vitro studies of the effects of the aminopyridines on muscle contractile performance.

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