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Modulation by presynaptic adenosine A_1 receptors of nicotinic receptor antagonist-induced neuromuscular block in the mouse

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Abstract

We have investigated how altering the activation of adenosine A_1 receptors modifies nicotinic receptor antagonist-induced fade of tetanic contractions in the mouse isolated hemi-diaphragm. Vecuronium-induced tetanic fade was attenuated by an adenosine A_1 receptor antagonist (8-cyclopentyl-1,3-dipropylxanthine, DPCPX, 10^{-7} M) and by an inhibitor of the synthesis of extracellular adenosine from ATP (α,β-methylene ADP, MeADP, 5×10^{-5} M). Conversely, vecuronium-induced tetanic fade was potentiated by an adenosine A_1 receptor agonist (N^6-cyclohexyladenosine, CHA, 10^{-7} M) and an inhibitor of the extracellular destruction of adenosine (*erythro*-9-[2-hydroxy-3-nonyl]adenine, EHNA, 10^{-4} M). The ability of an adenosine A_1 receptor antagonist to attenuate vecuronium-induced tetanic fade indicates that a component of this fade is due to endogenous adenosine. Further, the ability of the inhibitor of adenosine synthesis to attenuate vecuronium-induced tetanic fade indicates that this endogenous adenosine is derived from ATP. Hexamethonium-induced tetanic fade was also potentiated by increasing adenosine A_1 receptor activation, albeit with a higher concentration of CHA (10^{-4} M). However, unlike for vecuronium, hexamethonium-induced tetanic fade was not attenuated by reducing adenosine A_1 receptor activation. This latter observation suggests that the tetanic fade produced by hexamethonium and vecuronium does not share a common mechanism of action.

Keywords: Nicotinic receptor antagonist; Adenosine; Neuromuscular junction; Acetylcholine release

1. Introduction

Neuromuscular depression is an inability of the neuromuscular junction to sustain normal synaptic transmission during high frequency activation. In isolated skeletal muscle, neuromuscular depression produces tetanic fade, characterised as a waning of tension during periods of tetanic stimulation. Nicotinic receptor antagonists such as vecuronium and hexamethonium (Gibb and Marshall, 1986) and adenosine (Bowman, 1996) all produce tetanic fade at concentrations that have little effect on singly evoked twitch force. This neuromuscular depression has been attributed to a presynaptic action of the compounds on evoked acetylcholine release; adenosine by activating inhibitory presynaptic adenosine A_1 receptors (Redman and Silinsky, 1993, 1994) and nicotinic receptor antagonists by blocking presynaptic nicotinic acetylcholine autoreceptors (reviewed by Bowman et al., 1990; Prior et al., 1995).

Extracellular adenosine is present in large quantities at the neuromuscular synapse and it increases during stimulation (Smith, 1991). This adenosine comes from the hydrolysis by ecto-5′-nucleotidases of ATP co-released with acetylcholine from nerve endings and also directly from contracting muscle. It is inactivated by conversion to inosine by adenosine deaminase. What is not clear at present is the extent to which this endogenous adenosine contributes to the neuromuscular depression produced by nicotinic receptor antagonists. To investigate this we have studied how neuromuscular depression produced by two different nicotinic receptor antagonists is modulated by conditions that alter the prevailing synaptic concentration of adenosine or the activation state of presynaptic adenosine A_1 receptors. To assess neuromuscular depression we have used tetanic fade in the mouse isolated hemi-diaphragm/phrenic nerve preparation. Vecuronium and hexamethonium were chosen as examples of nicotinic receptor antagonists with selectivity for muscle-type and ganglionic-type nicotinic receptors respectively (Bowman and Webb, 1972; Durant et al., 1979). We have examined how four different modulators of adenosine A_1 receptor activa-

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tion alter nicotinic receptor antagonist-induced tetanic fade: (1) an adenosine A_1 receptor agonist; (2) an adenosine A_1 receptor antagonist; (3) an inhibitor of adenosine deaminase; and (4) an inhibitor of ecto-5′-nucleotidases. Our data suggest that nicotinic receptor antagonist-induced tetanic fade can occur by different mechanisms, which show varying sensitivities to the actions of adenosine. Further, they hint at the existence of a complex balance between the presynaptic effects of acetylcholine and adenosine at the mouse neuromuscular junction.

2. Materials and methods

2.1. Mouse hemi-diaphragm preparation

Hemi-diaphragm muscles with approximately 10 mm of their associated phrenic nerve were isolated from Balb/c mice (25 g) killed by CO_2 anaesthesia followed by exsanguination. Preparations were mounted in 10 or 25 ml tissue baths at 37°C in a standard physiological solution (see below). Resting tension was adjusted to give maximum developed force when the nerve was stimulated (typically around 1 g resting tension). The nerve was stimulated using a pair of silver wire electrodes connected to a Grass S88 stimulator via a Grass SIU5 stimulus isolation unit. Pulses of 50–100 μs duration at supra-maximal voltage (typically 10–15 V) were used to excite the nerve. Indirectly elicited tension responses were monitored using a force displacement transducer (Grass, FT03C) linked to an ink-writing flat-bed recorder (Grass, 79D).

2.2. Experimental protocols

Preparations were stimulated with a 2 s train of impulses at 50 Hz every 10 min with continuous 0.1 Hz stimulation between each train. The nicotinic receptor antagonist was not added to the preparation until twitch force at 0.1 Hz was constant and two consecutive identical 50 Hz tetani were seen. We chose to work with a fixed degree of tetanic fade (50%) which best allowed us to observe both potentiation and attenuation of the responses. The average concentrations required to produce 50% tetanic fade are shown in Table 1. In each preparation, the modulator of adenosine A_1 receptor activation was only applied once fade was constant for two consecutive tetanic responses. The time required to reach a stable level of tetanic fade was different for the two nicotinic receptor antagonists (Table 1). However, once tetanic fade was stable, the modulator of adenosine A_1 receptor activation was applied midway between two tetanic responses. The effects of the test compound on nicotinic receptor antagonist-induced tetanic fade were assessed after 25 min exposure. This was sufficient to produce the maximal effects of all test compounds and, in the absence of their addition, tetanic fade was constant over this length of time for both nicotinic receptor antagonists. Four different modulators of adenosine A_1 receptor activation were studied. They were: the selective adenosine A_1 receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10^{-7} M); the adenosine deaminase inhibitor, *erythro*-9-(2-hydroxy-3-nonyl)adenine HCl (EHNA, 10^{-4} M); the stable adenosine A_1 receptor agonist, N^6-cyclohexyladenosine (CHA, 10^{-7} M and 10^{-4} M) and the inhibitor of ecto-5′-nucleotidases, α,β-methylene ADP (MeADP, 5×10^{-5} M).

2.3. Data analysis and statistics

Twitch force and peak tetanic force were expressed as a percentage of the control value, recorded in the same preparation prior to the addition of any pharmacological agent. Tetanic fade was expressed as a percentage of peak tetanic force using the following equation: fade $= 100 \times (F_P - F_E)/F_P$, where F_P is the peak force of the tetanus and F_E is the force at end of the tetanus. To ensure stability of the data in the presence of the nicotinic receptor antagonists, records were rejected for analysis if there was more than 5% difference in any of the tension parameters determined 15 and 5 min prior to the addition of the test compound. For each experimental group useable data from between 6 and 10 preparations, from at least 3 animals were obtained. Presented data are mean and standard error of the mean (S.E.M.). Statistical testing was performed using either a one-sample or paired Student's *t*-test. In all cases the tests were performed two-tailed and significance was assumed if $P < 0.05$.

2.4. Drugs and solutions

Experiments were performed at 37°C in Krebs-Henseleit solution (mM): NaCl, 118; KCl, 5; $CaCl_2$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1; glucose, 11, gassed with 5% CO_2 in 95% O_2 to a pH of 7.2–7.4. DPCPX, CHA and EHNA were purchased from Semat Technical (UK) (St. Albans, UK). MeADP and hexamethonium bromide were purchased from Sigma (Poole, UK). Vecuronium bromide was the gift of Organon Scientific Development Group (Newhouse, UK). Stock solutions of DPCPX, CHA, EHNA and MeADP were made at 100–1000-times their final bath concentration in water (MeADP), water acidified with 1–2 drops of 1 M HCl per ml of stock (CHA, EHNA) or ethanol (DPCPX). Hexamethonium was prepared as a 1 M stock solution in water and vecuronium was prepared as a 10^{-3} M stock in 10 mM citric acid. The range of concentrations needed for 50% tetanic fade was $(1.75–6) \times 10^{-7}$ M for vecuronium and $(0.9–1.75) \times 10^{-3}$ M for hexamethonium. Maximum concentrations of citrate and ethanol in the tissue bath were 6×10^{-6} M and 1.7×10^{-3} M, respectively, and these concentrations had no effect on tension responses. All stock solutions were kept in the dark at 4°C while not in use, except for the DPCPX stock which was made fresh each day.

3. Results

3.1. Effects of nicotinic receptor antagonists on tension responses

Across all studies, the average tetanic fade produced by vecuronium and hexamethonium was similar, and close to 50% (Table 1). At the concentrations producing this degree of tetanic fade, both nicotinic receptor antagonists produced similar small (around 10%), but significant, reductions in the peak force of twitch responses at 0.1 Hz (Table 1). The reductions of twitch force we attribute to an inhibitory action of the compounds on the post-junctional muscle-type nicotinic receptors. There was a relatively much greater effect of the two nicotinic receptor antagonists on the peak force of 50 Hz tetanic contractions; both compounds depressed this equally by around 30–40% (Table 1). This greater effect on peak tetanic force than on twitch force presumably reflects a more complex inhibitory action of the compounds on tetani. Prior to the addition of the adenosine modulator, no variations were seen in the twitch and tetanic responses between the four different experimental groups, for either of the nicotinic receptor antagonists studied.

Table 1
Effects of nicotinic receptor antagonists on twitch force (at 0.1 Hz) and peak tetanic force and tetanic fade (at 50 Hz)

	Vecuronium	Hexamethonium
Number of preparations	28	26
Concentration (μM)	0.30 ± 0.02	1360 ± 40
Equilibration period (min)	85 ± 8	122 ± 8
Twitch force (% control)	91.1 ± 1.3 [a]	87.8 ± 2.4 [a]
Peak tetanic force (% control)	69.0 ± 2.6 [a]	62.9 ± 2.9 [a]
Tetanic fade (%)	51.7 ± 2.6	52.6 ± 3.4

All values are mean and S.E.M. Twitch force and peak tetanic force are expressed as a percentage of the control value recorded prior to the addition of the nicotinic receptor antagonist. [a] $P < 0.05$, one-sample Student's *t*-test versus 100%. All tension data, non-significant for vecuronium versus hexamethonium ($P > 0.05$, paired Student's *t*-test).

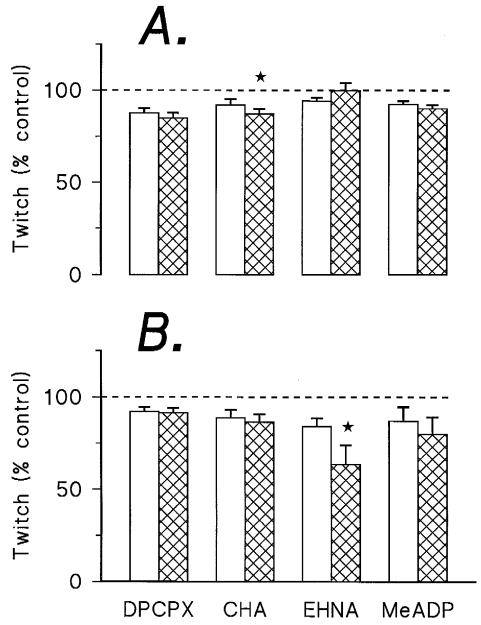

Fig. 1. Effects of modulators of adenosine A_1 receptor activation on twitch force (0.1 Hz) in the presence of vecuronium (A) or hexamethonium (B). Open columns show twitch force, expressed as a percentage control, recorded following exposure to vecuronium or hexamethonium. Cross-hatched columns show twitch force in the same preparations following a subsequent 25 min exposure to one of the four test compounds (see Section 2 for details of compounds). Each pair of columns shows the mean and S.E.M. of data from 6–10 different preparations. In all cases, the nicotinic receptor antagonist produced a small, but statistically significant decrease in twitch force ($P < 0.05$, one-sample Student's *t*-test versus 100%). DPCPX (10^{-7} M) and MeADP (5×10^{-5} M) were without further effect on twitch force with either nicotinic receptor antagonist. CHA (10^{-7} M) further reduced twitch force in the presence of vecuronium but not hexamethonium while EHNA (10^{-4} M) further reduced twitch force in the presence of hexamethonium but not vecuronium. All statistically significant differences ($P < 0.05$, paired Student's *t*-test) are indicated by stars.

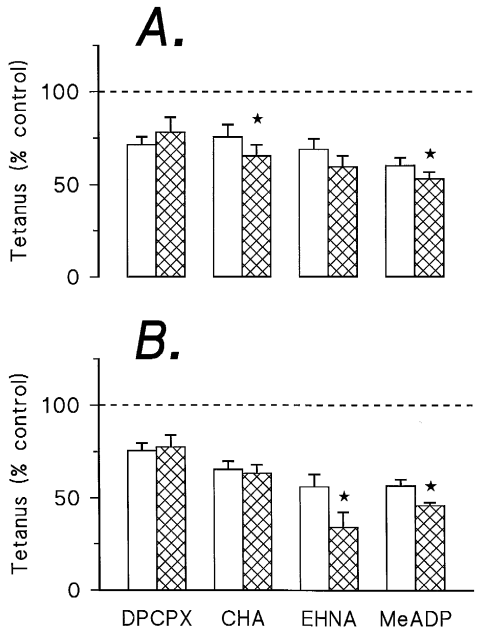

Fig. 2. Effect of modulators of adenosine A_1 receptor activation on the peak force of 50 Hz tetanic contractions in the presence of vecuronium (A) or hexamethonium (B). Open columns show peak tetanic force, expressed as a percentage control, recorded following exposure to vecuronium or hexamethonium. Cross-hatched columns show peak tetanic force in the same preparations following a subsequent 25 min exposure to one of the four test compounds (see Section 2 for details of compounds). Each pair of columns shows the mean and S.E.M. of data from 6–10 different preparations. In all cases, the nicotinic receptor antagonist produced a significant decrease in the peak force of 50 Hz tetani contractions ($P < 0.05$, one-sample Student's *t*-test versus 100%). DPCPX (10^{-7} M) was without further effect on peak tetanic force with either nicotinic receptor antagonist while MeADP produced a further significant reduction in peak tetanic force with both compounds. CHA (10^{-7} M) further reduced peak tetanic force in the presence of vecuronium but not hexamethonium while EHNA (10^{-4} M) further reduce peak tetanic force in the presence of hexamethonium but not vecuronium. All statistically significant differences ($P < 0.05$, paired Student's *t*-test) are indicated by stars.

3.2. Modulation of twitch force and peak tetanic force

The adenosine A_1 receptor agonist (CHA, 10^{-7} M) produced a small, but significant, decrease in the peak force of twitch responses recorded in the presence of vecuronium (Fig. 1A). In contrast, the other three compounds (DPCPX, 10^{-7} M; EHNA, 10^{-4} M; MeADP, 5×10^{-5} M) had no effect on twitch force in the presence of vecuronium (Fig. 1A). In hexamethonium, only EHNA (10^{-4} M) reduced twitch force (Fig. 1B). However, with hexamethonium, although 10^{-7} M CHA had no effect on twitch force, 10^{-4} M CHA did produce a significant depression of this parameter. The four modulators of adenosine A_1 receptor activation produced a broadly similar pattern of results on the peak tetanic force (Fig. 2) as seen for the twitch force (Fig. 1). Thus DPCPX (10^{-7} M) had no effect on peak tetanic tension with either nicotinic receptor antagonist, CHA (10^{-7} M) depressed the peak tetanic force in the presence of vecuronium but not hexamethonium and EHNA (10^{-4} M) depressed peak tetanic force in the presence of hexamethonium but not vecuronium. However, MeADP (5×10^{-5} M), which had no effect on twitch force with either nicotinic receptor antagonist, produced a significant depression of peak tetanic force in the presence of both vecuronium and hexamethonium. As for twitch force, in the presence of hexamethonium, although 10^{-7} M CHA was without effect on peak tetanic force, 10^{-4} M CHA caused a marked depression of this parameter.

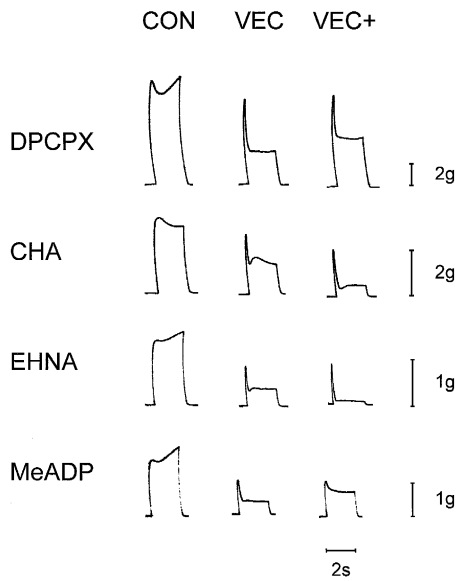

Fig. 3. Representative examples of tetanic contractions recorded from the mouse hemi-diaphragm preparation in response to a 2 s period of 50 Hz motor nerve stimulation. Columns are: control responses (left); responses showing approximately 50% vecuronium-induced tetanic fade (centre) and responses showing vecuronium-induced fade 25 min following application of an adenosine modulator (right). Each row shows responses from the same muscle preparation. Responses shown in the right-hand column were recorded in the presence of 10^{-7} M DPCPX; 10^{-7} M CHA; 10^{-4} M EHNA and 5×10^{-5} M MeADP. Note the attenuation of vecuronium-induced tetanic fade by DPCPX and MeADP and the potentiation of tetanic fade by CHA and EHNA.

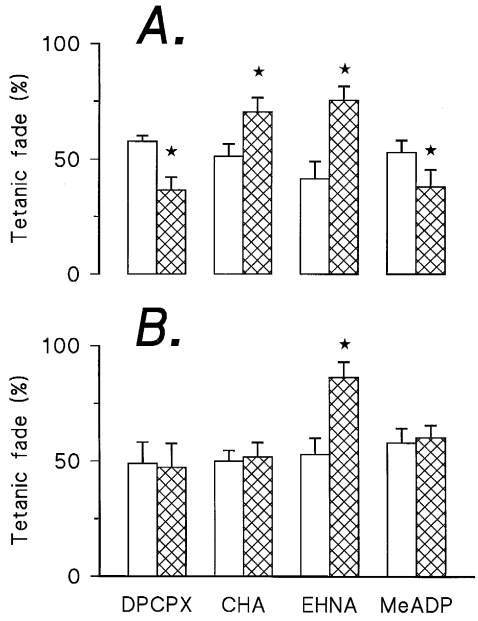

Fig. 4. Effect of modulators of adenosine A_1 receptor activation on tetanic fade produced by vecuronium (A) or hexamethonium (B). Open columns show tetanic fade recorded following exposure to vecuronium or hexamethonium. Cross-hatched columns show tetanic fade in the same preparations following a subsequent 25 min exposure to one of the four test compounds (see Section 2 for details of compounds). Each pair of columns shows the mean and S.E.M. of data from 6–10 different preparations. In all cases, the nicotinic receptor antagonist produced approximately 50% tetanic fade. CHA (10^{-7} M) and EHNA (10^{-4} M) significantly potentiated vecuronium-induced tetanic fade. EHNA (10^{-4} M), but not CHA (10^{-7} M), attenuated hexamethonium-induced tetanic fade. DPCPX (10^{-7} M) and MeADP (5×10^{-5} M) significantly attenuated vecuronium-induced tetanic fade but had no effect on hexamethonium-induced tetanic fade. Statistically significant differences ($P < 0.05$, paired Student's *t*-test) are indicated by stars.

3.3. Modulation of tetanic fade

The 50% fade of 50 Hz tetanic contractions produced by vecuronium was significantly potentiated by both CHA (10^{-7} M) and EHNA (10^{-4} M). In contrast, it was significantly attenuated by both DPCPX (10^{-7} M) and MeADP (5×10^{-5} M). Representative tetani showing the effects of the adenosine modulators on vecuronium-induced fade of 50 Hz tetanic contractions are shown in Fig. 3 and averaged tetanic fade data for all the vecuronium studies are shown in Fig. 4A. With hexamethonium, EHNA (10^{-4} M) produced similar changes in tetanic fade to those seen with vecuronium – i.e. a marked potentiation (Fig. 4B). However, CHA (10^{-7} M) had no potentiating effect on hexamethonium-induced tetanic fade (Fig. 4B). Elevating the concentration of CHA to 10^{-4} M did lead to a potentiation of hexamethonium-induced tetanic fade: hex-

amethonium, 53.0 ± 7.2%; hexamethonium and CHA (10^{-4} M), 93.2 ± 3.4 ($n = 6$, $P < 0.05$, paired Student's *t*-test). This was accompanied by significant decreases in the peak force of both twitches and tetani. Finally, in direct contrast to vecuronium-induced tetanic fade, neither MeADP (5×10^{-5} M) nor DPCPX (10^{-7} M) had an effect on hexamethonium-induced tetanic fade (Fig. 4B).

4. Discussion

Our data suggest that there is an interaction between the effects of endogenous adenosine and nicotinic receptor antagonists on neuromuscular transmission in the mouse. Thus, vecuronium-induced tetanic fade was partially reversed by an adenosine A_1 receptor antagonist and an inhibitor of ecto-5′-nucleotidases. In contrast, an adenosine deaminase inhibitor and an adenosine A_1 receptor agonist both potentiated vecuronium-induced tetanic fade. However, reducing adenosine A_1 receptor activation did not attenuate tetanic fade produced by hexamethonium and a higher concentration of the adenosine A_1 receptor agonist was required to potentiate the tetanic fade produced by hexamethonium compared with vecuronium. These results suggest that the effects of the two nicotinic receptor antagonists, though superficially identical in appearance, may be a due to different underlying physiological mechanisms. In electrophysiological studies both vecuronium (Tian et al., 1994) and hexamethonium (Tian et al., 1996) have presynaptic effects to increase the frequency dependence of acetylcholine release – i.e. they increase the ratio of acetylcholine released at low and high frequencies of nerve stimulation. This effect is believed to involve the inhibition of presynaptic nicotinic receptors and, combined with a post-junctionally mediated reduction in safety factor, would cause tetanic fade. Given this basis for tetanic fade it is not difficult to envisage a mechanism whereby the effects of nicotinic receptor antagonists on tetani are modulated by the level of presynaptic adenosine A_1 receptor activation (see below). However, the inability of DPCPX and MeADP to attenuate hexamethonium-induced tetanic fade suggests that the mechanism underlying tetanic fade with this compound is not modulation of acetylcholine release. Rather, it is likely that, at the high concentrations required to block neuromuscular transmission (around 10^{-3} M), hexamethonium produces marked post-junctional endplate ion channel block. This action of hexamethonium has been well characterised at the neuromuscular junction and is seen at a similar range of concentrations to those used here (Milne and Byrne, 1981). Ion channel block is a use-dependent phenomenon and consequently leads to fade of high frequency tetanic contractions. However, unlike tetanic fade produced by a depression of acetylcholine release, tetanic fade produced by ion channel block would not be attenuated by treatments that elevate acetylcholine release.

The decreases in the peak force of twitches and tetani produced by CHA (10^{-7} M) in the vecuronium studies and by CHA (10^{-4} M) and EHNA (5×10^{-5} M) in the hexamethonium studies can be accounted for in terms of an adenosine A_1 receptor-mediated depression of acetylcholine release even at low frequencies of stimulation. However, why EHNA had no effect on these parameters in the vecuronium studies is at present unclear. The depression of peak tetanic force by MeADP is presumably due to a different mechanism than the inhibition of presynaptic adenosine A_1 receptor activation. The exact reason for this effect of MeADP is uncertain but one possibility, given the relatively high concentrations of the compound used, is that it represents a non-specific inhibitory action of the compound such as end-plate ion channel block. Alternatively, the depression of peak tetanic amplitude by MeADP could reflect the loss of a tonic effect of adenosine on excitatory presynaptic adenosine A_{2A} receptors that, it is postulated, are preferentially activated by adenosine formed from the metabolism of synaptically released ATP (Cunha et al., 1996). The fact that MeADP reduced the peak tetanic force but not twitch force suggests that tetani may be the more sensitive measure of neuromuscular compromise.

It is unlikely that vecuronium, by inhibiting presynaptic nicotinic receptors could increase the activation of presynaptic adenosine A_1 receptors by endogenous adenosine. Therefore, the ability of DPCPX to attenuate vecuronium-induced tetanic fade suggests that there must be some tonic activation of these receptors under normal circumstances – i.e. endogenous adenosine has a role in the neuromuscular depression produced by vecuronium. Further, the fact that MeADP is also effective in reversing vecuronium-induced fade is consistent with the notion that this endogenous adenosine is derived from AMP, probably originated from ATP released from the nerve terminal by stimulation. Given that the reversal of vecuronium-induced tetanic fade by DPCPX and MeADP is due to the inhibition of an inhibitory system it is perhaps not surprising that the maximum effect is limited and only partial reversal is seen.

One model that explains our observations is that acetylcholine, acting through presynaptic nicotinic receptors and adenosine, acting through presynaptic adenosine A_1 receptors have opposing, but independent, actions on some part of the acetylcholine release process. This could involve either intracellular cAMP (increased by acetylcholine and decreased by adenosine) or a presynaptic Ca^{2+} current (reduced by adenosine and enhanced by acetylcholine). Indeed, there is evidence to suggest that both suxamethonium, a potent cholinoceptor agonist, and PSPT (a non-selective adenosine receptor antagonist) can increase the Ca^{2+} current associated with transmitter release in mouse motor nerve endings (Braga et al., 1994; E.G. Rowan, personal communication). This model predicts that adenosine agonists, in the absence of a nicotinic receptor antagonist, would cause neuromuscular depression. Demonstrating this has proved elusive. Adenosine only affects twitch

force in mammalian skeletal muscle preparations where neuromuscular transmission has been partially blocked through the use of Mg^{2+} to reduce acetylcholine release or a nicotinic receptor antagonist to reduce post-junctional sensitivity to acetylcholine (Ribeiro and Sebastião, 1985; Sebastião and Ribeiro, 1988; Nagano et al., 1992). Similarly, we saw that CHA (10^{-4} M) had no effect in muscles not exposed to a nicotinic receptor antagonist. These do not prove that adenosine is without effect in uncompromised preparations but simply indicate that any effect of the compound on acetylcholine release may be masked by either the presence of the large safety factor for transmission or the unhindered presynaptic effect of endogenous acetylcholine. Unfortunately, existing electrophysiological studies of the effects of adenosine on evoked acetylcholine release do not completely resolve the uncertainty since the majority of these studies have employed a nicotinic receptor antagonist such as tubocurarine to immobilise the muscle preparation during intracellular microelectrode recording (see, e.g., Redman and Silinsky, 1993, 1994). An exception is the study of Singh et al. (1986) in which, using mouse hemi-diaphragm muscles paralysed by cutting the muscle fibres, it was demonstrated that adenosine agonists do produce a decrease in acetylcholine release evoked by high frequency stimulation. Finally, an independent-action model would predict that tetanic fade would only be attenuated by decreased adenosine A_1 receptor activation if it occurred as a result of a change in acetylcholine release. However, the model would not preclude the ability of enhanced adenosine A_1 receptor activation to enhance tetanic fade, irrespective of the mechanism by which it was produced.

In conclusion, in the mouse the neuromuscular depression (seen as tetanic fade) produced by vecuronium, but not hexamethonium, is attenuated by reducing the activation of adenosine A_1 receptors. We have accounted for the differences between vecuronium and hexamethonium in terms of a presynaptic effect of the former (decreased acetylcholine release) and a post-junctional effect of the latter (ion channel block). Our results for vecuronium are consistent with current theories relating to the presynaptic effects of adenosine and acetylcholine on motor endings. They would suggest that a component of the presynaptic effect of vecuronium is specifically dependent on the activation of presynaptic inhibitory adenosine A_1 receptors by endogenous adenosine. However, tension studies do not allow us to determine the complex processes underlying the presynaptic effects of endogenous adenosine and nicotinic receptor antagonists such as vecuronium. More detailed studies of acetylcholine release using radio-isotopic and electrophysiological approaches will be needed to conclusively identify the mechanisms involved.

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