Prejunctional modulation of acetylcholine release from the skeletal neuromuscular junction: link between positive (nicotinic)- and negative (muscarinic)-feedback modulation

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1 Presynaptic receptor-mediated modulation of stimulation-evoked [³H]-acetylcholine([³H]-ACh) release from the neuromuscular junction was studied in the region of the mouse hemidiaphragm which contains the motor endplates, and which can easily be loaded with [³H]-choline. This method made it possible to detect exclusively the $[Ca^{2+}]_o$ -dependent, quantal release of [³H]-ACh in response to axonal stimulation.

2 Atropine enhanced, and non-depolarizing muscle relaxants ((+)-tubocurarine, pancuronium and picrocuronium) reduced, the release of [³H]-ACh evoked by high frequency trains of stimulation (50 Hz, 40 shocks) of the phrenic nerve. The effect of (+)-tubocurarine was frequency-dependent as at 5 Hz (40 shocks) it was less effective than at 50 Hz. The resting release of [³H]-ACh was not affected by these compounds. These findings indicate that ACh released into the synaptic gap by axonal firing reaches a concentration sufficient to influence its own release by a prejunctional effect.

3 The anticholinesterase, physostigmine sulphate, enhanced the release of [³H]-ACh in a concentration-dependent manner. This effect was mediated via prejunctional nicotinic receptor stimulation: (+)-tubocurarine, pancuronium and picrocuronium completely prevented the effect of physostigmine.

4 When the prejunctional nicotinic and muscarinic receptors were stimulated by a high concentration of extracellular ACh which had accumulated in the junctional gap in the presence of physostigmine, atropine did not influence the evoked release of [³H]-ACh. However, when the effect of endogenous ACh on nicotinic receptors was prevented by (+)-tubocurarine, atropine enhanced the release.

5 It is concluded that quantally-released ACh from motor endplates is subject to prejunctional automodulation: (a) ACh facilitates its own release via an effect on prejunctional nicotinic receptors (positive feedback), (b) ACh release is reduced by an action on muscarinic receptors. When the nicotinic receptor-mediated facilitation is fully operative, the muscarinic receptor-mediated negative feedback is much less effective. It is supposed that there is a link between the two feedback mechanisms possibly at the level of the second messenger system(s).

Introduction

Electrophysiological (Hubbard *et al.*, 1969; Blaber, 1973; Gibb & Marschall, 1983; Matzner *et al.*, 1988) and pharmacological (Bowman & Webb, 1976; Bowman, 1980a, b; cf. Bowman *et al.*, 1988) studies have provided evidence to suggest that (+)-tubocurarine might, in addition to its post-junctional actions, exert a prejunctional effect, and inhibit the evoked release of acetylcholine (ACh). Bowman (1980b) suggested that ACh facilitates its own release

through a positive feedback mechanism at high frequency nerve stimulation. The radioisotope method (Foldes *et al.*, 1984) has made it possible to measure the Ca^{2+} -dependent quantal release of ACh from motor nerves at the neuromuscular junction independently of non-quantal resting release (Somogyi *et al.*, 1987; Somogyi & Vizi, 1987) which is Ca -independent.

In recent years, neurochemical evidence has been obtained (Halank *et al.*, 1985; Vizi *et al.*, 1985; 1987;

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Wessler *et al.*, 1986; Somogyi & Vizi, 1987; Somogyi *et al.*, 1987) showing that non-depolarizing muscle relaxants, in addition to post-junctional blockade, exert prejunctional actions. (+)-Tubocurarine (Wessler *et al.*, 1986; 1987a; Vizi *et al.*, 1987), pancuronium and hexamethonium (Vizi *et al.*, 1987) reduced the stimulation-evoked release of ACh from motor nerve endings of diaphragm. In agreement with these findings, 1,1-dimethyl-4-phenyl piperazinium (DMPP), a nicotinic receptor agonist, was shown to enhance the release of ACh from the rat hemidiaphragm preparation (Wessler *et al.*, 1986). It was shown that nicotinic agonists as well as anticholinesterase agents generated antidromic axonal firing which was inhibited by nicotinic antagonists (cf. Bowman, 1980a). The effect of anticholinesterase may have been caused by an accumulation of spontaneously released ACh (Katz & Miledi, 1977) which activated the prejunctional nicotinic receptors. No electrophysiological evidence is available to suggest that prejunctional muscarinic stimulation reduces chemical transmission (Beranek & Vyskocil, 1967). However, it has been shown that the muscarinic receptor agonist oxotremorine reduced (Somogyi *et al.*, 1987), and the antagonist atropine enhanced (Somogyi *et al.*, 1987; Wessler *et al.*, 1987b) the release of ACh evoked by electrical stimulation. This suggests that negative feedback modulation mediated via muscarinic receptors occurs at the neuromuscular junction (Somogyi *et al.*, 1987; Wessler *et al.*, 1987b).

In this study an attempt was made to investigate the possible interaction between muscarinic autoinhibition and nicotinic receptor-mediated positive feedback modulation of ACh release at prejunctional axon terminals of the mouse hemidiaphragm preparation. In view of the multiple effects of physostigmine (cf. Bowman, 1980a) on neuromuscular transmission in some experiments the cholinesterase activity was inhibited with physostigmine sulphate to amplify the effect of released ACh on prejunctional axon terminals. In this way the role of prejunctional cholinceptors was more easily studied.

Methods

Male Swiss-Webster mice, weighing 25–30 g, were decapitated. Both hemidiaphragms were prepared (Somogyi *et al.*, 1987) and the phrenic nerves were cut about 1 mm from the muscle. Strips with the innervated region containing the phrenic nerve terminals about 2 mm to the right and left of the insertions of the phrenic nerves were mounted in separate 2 ml baths in Krebs solution gassed with a mixture of 95% O₂ and 5% CO₂ at 37°C. The composition of the Krebs solution was (mmol l⁻¹): NaCl 113,

KCl 4.7, CaCl₂ 2.5, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11.5.

The preparations were incubated for 60 min in a solution containing 185 kBq ml⁻¹ methyl [³H]-choline (Somogyi *et al.*, 1987). To facilitate the uptake of [³H]-choline and the synthesis of [³H]-ACh the preparation was stimulated (square wave pulses, supramaximal, 0.2 ms duration, 1 Hz) during incubation. After incubation the preparations were transferred to a 1 ml bath and superfused continuously with solution at a rate of 2 ml min⁻¹ for 90 min to wash out excess radioactivity. Subsequently, the perfusion rate was reduced to 0.5 ml min⁻¹, and 10 µmol l⁻¹ hemicholinium-3 was added to the perfusing solution to prevent reabsorption of [³H]-choline originating from the hydrolysis of [³H]-acetylcholine released by electrical stimulation. After the washout period, 3 min aliquots of the effluent were collected by an automated fraction collector. The preparations were stimulated for 3 min on two occasions, the times depending on the experimental schedule. This endplate-rich preparation of the phrenic nerve-hemidiaphragm synthesizes [³H]-ACh from [³H]-choline (Wessler & Steinlein, 1987) and the newly synthesized [³H]-ACh (Somogyi *et al.*, 1987) is released by electrical stimulation.

Measurement of radioactivity

The tritium content of the tissue was determined after perfusion by placing the preparation into 1 ml Soluene (Packard) and an aliquot of 0.1 ml was used for scintillation counting.

Ten ml of scintillation fluid containing toluene + Triton X-100 (3:1, v/v) were added to each 3 min aliquots of perfusate. The activity of the samples was measured in a LKB scintillation beta spectrometer. The radioactivity was expressed as Bq 3 min⁻¹ g⁻¹ wet weight of the tissue determined at the end of the experiment. The effect of drugs was expressed by changes in the S2/S1 ratio. The calculation of S2/S1 ratio was based on fractional release of radioactivity.

Stimulation

The preparations were stimulated by square wave pulses of 0.2 ms duration at a voltage five times higher than that which produced maximal contractions (20 V, 50 mA). Unless otherwise stated, trains of 40 impulses (50 Hz) were administered every 10 s for 3 min through a pair of platinum electrodes placed at the top and the bottom of the organ bath (field stimulation). The preparations were stimulated twice for 3 min each starting at the 6th (S1) and 36th (S2) min.

Figure 1 The effect of physostigmine (5×10^{-5} M) on the release of radioactivity from the endplate rich region of neuromuscular junction (mouse hemidiaphragm) in response to electrical stimulation. (a) Control experiments. Periods of stimulation (50 Hz, trains of 40 shocks) were applied every 10 s as indicated by the horizontal bars. (b) Effect of physostigmine sulphate (5×10^{-5} M). Each point represents the mean ($n = 3$ (a) or 4(b)) and vertical lines indicate s.e.mean.

Statistics

Paired *t* test, Student's *t* test, or one way analysis of variance was used, and $P < 0.05$ was accepted as significant.

Drugs

Methyl- $[^3\text{H}]$ -choline ($2.89 \text{ TBq mmol}^{-1}$, Amersham, Arlington Heights, IL, U.S.A.), atropine sulphate (Burroughs Wellcome, Burlingame, CA, U.S.A.), hemicholinium-3 (Aldrich, Steinheim, F.R.G), pancu-

Table 1 Effect of (+)-tubocurarine (Tc) and atropine on $[^3\text{H}]$ -acetylcholine ($[^3\text{H}]$ -ACh) release from the endplate-rich portion of mouse hemidiaphragm

Drugs	n	Effect on $[^3\text{H}]$ -ACh release (S2/S1)	
		50 Hz	5 Hz
Control	(8)	0.86 ± 0.04	0.91 ± 0.07
Tc, $50 \mu\text{M}$	(4)	$0.41 \pm 0.07^*$	$0.65 \pm 0.02^*$
Atropine, $1 \mu\text{M}$	(4)	$2.52 \pm 0.30^*$	$1.46 \pm 0.16^*$
Tc, $50 \mu\text{M}$ + atropine, $1 \mu\text{M}$	(4)	$2.81 \pm 0.19^*$	$1.51 \pm 0.11^*$

The drugs were administered 12 min before S2. Between the two stimulation periods (50 Hz, 40 shocks every 10 s for 3 min or 5 Hz 40 shocks every 10 s for 3 min) 30 min elapsed. Significance: ANOVA followed by Dunn's test. *Significant difference ($P < 0.05$) between the control values and the value obtained in the presence of drug. Note that the differences between the effect of drugs on 50 Hz and 5 Hz are significant. Data show means $\pm$ s.e.mean (n = number of experiments).

ronium (Pavulon, Organon) and pipercuronium (Gedeon Richter, Budapest, Hungary) were used. The chemicals used for the Krebs solution were of analytical grade from Fischer (New York, U.S.A.).

Results

Resting and stimulation-evoked release of $[^3\text{H}]$ -ACh

After loading, the preparation contained $225,600 \pm 20,450 \text{ Bq g}^{-1}$ ^3H and the resting release was $3450 \pm 230 \text{ Bq g}^{-1}$ in 3 min (16 experiments). Electrical field stimulation (50 Hz, 40 shocks every 10 s for 3 min) released $3680 \pm 170 \text{ Bq g}^{-1}$ $[^3\text{H}]$ -ACh. The stimulation was repeated within 30 min (S2) and the release was $3015 \pm 195 \text{ Bq g}^{-1}$ ($n = 8$). The S2/S1 ratio was constant (Figure 1a). Therefore, the effect of different drugs was evaluated from changes in S2/S1 ratios. (+)-Tubocurarine ($50 \mu\text{M}$) reduced the ratio, (i.e. the release of $[^3\text{H}]$ -ACh), while atropine ($1 \mu\text{M}$) enhanced the release of $[^3\text{H}]$ -ACh (Table 1). When both receptors were inhibited the release of $[^3\text{H}]$ -ACh was higher than in control conditions which suggests that the release of ACh is modulated by both nicotinic and muscarinic receptors. In order to enhance the biophase concentration of ACh in the junctional cleft and thereby amplify the possible prejunctonal effect of ACh on nicotinic and muscarinic receptors an anticholinesterase, physostigmine sulphate, was applied. Figure 1b shows the effect of physostigmine on evoked release of $[^3\text{H}]$ -ACh. In the presence of physostigmine the

Figure 2 Effect of physostigmine sulphate on the stimulation-evoked release of [^3H]-acetylcholine from the endplate-rich region of mouse-hemidiaphragm preparation. Physostigmine was added in different concentrations between S1 and S2 as indicated in Figure 1b. Stimulation: trains of 40 stimuli (50 Hz), with an interval of 10 s between trains, were applied. Stimulation period: 3 min. Each point represents the mean and vertical lines indicate s.e.mean.

release was significantly higher ($S2/S1 = 1.46 \pm 0.22$, $P < 0.01$) than in its absence ($S2/S1 = 0.89 \pm 0.08$, $n = 4$). The effect of physostigmine on the release of [^3H]-ACh was concentration-dependent (Figure 2).

Interaction between muscarinic and nicotinic presynaptic modulation

Figure 3 shows that (+)-tubocurarine ($2 \times 10^{-5} \text{ M}$) significantly reduced the release of [^3H]-ACh enhanced by physostigmine ($S2/S1 = 0.37 \pm 0.12$). That this reduction in [^3H]-ACh release is mediated not only via removal of nicotinic receptor stimulation but by concomitant stimulation of muscarinic

Figure 3 Inhibitory effect of (+)-tubocurarine ($2 \times 10^{-5} \text{ M}$) on [^3H]-acetylcholine release enhanced by physostigmine ($5 \times 10^{-5} \text{ M}$). Two periods of stimulation (50 Hz, trains of 40 shocks) were applied as indicated by the horizontal bars. Each point represents the mean ($n = 4$) and vertical lines indicate s.e.mean.

receptors, is indicated by the finding (Figure 3) that atropine (10^{-6} M) completely reversed this effect of (+)-tubocurarine ($S2/S1 = 2.70 \pm 0.28$). Pancuronium (10^{-5} M) and pitecuronium (10^{-5} M) also prevented the effect of physostigmine. The $S2/S1$ ratio in the presence of pancuronium was 0.64 ± 0.08 ($n = 3$) and with pitecuronium 0.51 ± 0.11 ($n = 4$). These values are significantly ($P < 0.01$ in both cases) smaller than that obtained in the absence of muscle relaxants, 1.61 ± 0.12 ($n = 4$).

In the presence of physostigmine, atropine (10^{-6} M) failed to affect the release of [^3H]-ACh. However, under control conditions atropine (10^{-6} M) enhanced the release from 0.83 ± 0.09 to 2.52 ± 0.39 (Table 1).

Discussion

Dale *et al.* (1936) found that the amount of ACh released into the solution perfusing striated muscle was not diminished when the muscles were completely paralysed by curare. They concluded that curare had principally a postjunctional action. Since then several attempts have been made to study the prejunctional effect of (+)-tubocurarine (Chang *et al.*, 1967; Fletcher & Forrester, 1975), but until now attempts to demonstrate the inhibitory effect of (+)-tubocurarine on the evoked release of ACh by neurochemical methods (i.e. assay of the released ACh) have been unsuccessful. This was always considered a strong argument in favour of a purely postjunctional action of curare. In all of these studies the total (quantal + non-quantal) release of ACh was measured in the presence of an anticholinesterase. The failure to demonstrate any changes in the evoked, quantal release of ACh by (+)-tubocurarine or muscarinic stimulation may have been due to the fact that most of the endogenous ACh released from mammalian muscle is released in a non-quantal, Ca-independent way (Mitchell & Silver, 1963; Katz & Miledi, 1977; Vizi & Vyskocil, 1979). Very recently a sensitive radioactive method that measures selectively the evoked (Ca-dependent) release of ACh from the mouse (Somogyi *et al.*, 1987) or rat (Wessler & Kilbinger, 1986) phrenic nerve-hemidiaphragm preparation *in vitro* has made it possible to measure the effect of drugs on the quantal release of labelled ACh from motor nerve endings. The mouse hemidiaphragm preparation (Somogyi *et al.*, 1987) seems to be a suitable tissue for studying quantally released ACh, because it is both rich in endplates (Waser *et al.*, 1978) and thin which allows easy access to drugs as well as diffusion of ACh into the perfusing fluid. The signal:noise (evoked:resting release) ratio in this preparation is much higher than that obtained in the rat hemidiaphragm (Wessler & Kilbinger, 1986). Based on electrophysiological (cf. Gibb &

Marshall, 1983) and pharmacological (Bowman & Webb, 1976; Bowman, 1980a; Bowman *et al.*, 1988) data, it was suggested that there is a prejunctional nicotinic receptor-mediated facilitation (mobilization) of ACh release by a positive feedback mechanism.

In this study neurochemical evidence was obtained that non-depolarizing muscle relaxants (+)-tubocurarine, pancuronium, pipecuronium reduced the stimulation-evoked release of [3 H]-ACh, and that atropine, a muscarinic receptor antagonist, enhanced it. These compounds did not affect the resting release in the absence of nerve stimulation. In a different set of experiments physostigmine was used to enhance the biophase concentration of ACh in the junctional gap, thereby amplifying the possible effect of ACh on prejunctional receptors. Physostigmine, by itself, enhanced the release of [3 H]-ACh. When cholinesterase is inhibited, ACh is allowed to make multiple interactions with the cholinergic receptors before escaping by diffusion from the receptor sites. Enhancement of ACh release by physostigmine was completely inhibited by (+)-tubocurarine and other non-depolarizing muscle relaxants (pancuronium, pipecuronium) which indicates that the effect of physostigmine was mediated via prejunctional nicotinic receptors. The possibility that physostigmine *per se* might increase the amount of transmitter released by each nerve impulse cannot be ignored.

As far as the muscarinic negative feedback modulation is concerned, the literature offers very controversial data. While Abbs & Joseph (1981) have shown that oxotremorine reduces and atropine enhances the release of ACh from the neuromuscular junction, others (Gundersen & Jenden, 1980; Haggblad & Heilbronn, 1983) failed to find such an effect. In addition, electrophysiological (Beranek & Vyskocil, 1967) and histochemical data (Gillberg *et al.*, 1984) oppose the presence of muscarinic receptors at the neuromuscular junction. Wessler *et al.* (1987b) found that the stimulation by oxotremorine of muscarinic receptors resulted in a biphasic response:

in low concentrations (10 nM) oxotremorine enhanced whereas in higher concentrations (1 μ M) it inhibited the release of ACh. These data and our earlier observations that oxotremorine reduces the release of ACh (Vizi *et al.*, 1985; Somogyi *et al.*, 1987) suggest that the release of ACh from the neuromuscular junction is modulated by muscarinic receptors. However, in our study atropine enhanced the release evoked by electrical stimulation and attenuated the reduction of release following (+)-tubocurarine administration. Atropine did not change the resting release of ACh. It is generally accepted that inhibition of presynaptic muscarinic autoreceptors enhances (cf. Kilbinger, 1977; Vizi, 1984) ACh release in both central (Szerb & Somogyi, 1973) and peripheral cholinergic synapses (Szerb & Somogyi, 1973; Vizi *et al.*, 1984; Baux & Tauc, 1987), whereas muscarinic receptor agonists (ACh, oxotremorine) inhibit the release. Therefore, the concept of a muscarinic feedback modulation of ACh release may be extended to the neuromuscular junction.

It is interesting to consider how our results obtained with different frequencies compare with already published data. It is known that (+)-tubocurarine is more effective at high frequencies of stimulation (cf. Bowman, 1980a) and in our experiments it was more effective in reducing ACh release at 50 Hz than at 5 Hz.

The data presented here also show that the modulation of ACh release at the neuromuscular junction is balanced through nicotinic and muscarinic autoreceptors. If it is assumed that nicotinic autoreceptors influence ACh release, then while positive feedback modulation is operative, inhibition of negative feedback modulation should not have any significant action. This was the case in our experiments. Atropine had no effect on the release evoked by physostigmine, i.e. when the nicotinic positive feedback modulation was fully operative. Therefore, we may conclude that positive feedback is dominant in the modulation of ACh release.

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