Selective antagonism to succinylcholine-induced depolarization by α -bungarotoxin with respect to the mode of action of depolarizing agents

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- 1 The interactions of α -bungarotoxin or tubocurarine with the neuromuscular block and endplate depolarization induced by succinylcholine (SCh) in the phrenic nerve-diaphragm preparation of mice were studied in order to elucidate the role of depolarization by SCh in the neuromuscular blockade.
- 2 The SCh concentrations required to depress the indirect twitch response by 20% and the evoked endplate potential in cut muscle preparations by 80% were 10 μ M and 6 μ M, respectively, while only 2 μ M SCh was needed to induce maximal endplate depolarization from -80 mV to about -60 mV.
- 3 SCh blocked the neuromuscular transmission synergistically with either α -bungarotoxin or tubocurarine. There was an initial partial reversal of the neuromuscular inhibition caused by tubocurarine, but not that by α -bungarotoxin.
- 4 α -Bungarotoxin (0.025 μ M) antagonized SCh (10 μ M)-induced depolarization more effectively than it depressed miniature endplate potentials and the antagonism was insurmountable by increasing SCh concentration. By contrast, tubocurarine preferentially depressed miniature endplate potentials and antagonized SCh-depolarization competitively.
- 5 The above difference was attributed to the irreversible nature of α -bungarotoxin binding to acetylcholine receptors, to the slow diffusion of the toxin molecule into the synaptic cleft and thus to the more rapid binding with perijunctional receptors compared with junctional ones.
- 6 It is concluded that the sustained depolarization of the endplate by SCh results largely from an action on the perijunctional receptor in mice and, unlike cats, the neuromuscular block by SCh is not due to the depolarization *per se* but rather to a direct attenuation of endplate potential.

Introduction

The mechanism of neuromuscular blockade by agonist-type depolarizing agents has been disputed for a long time (for comprehensive literatures, see Zaimis, 1976; Zaimis & Head, 1976; Colquhoun, 1986). It is generally attributed, at least for the initial phase of action, to the inactivation of voltage-dependent sodium channels at the endplate zone resulting from a sustained depolarization (Burns & Paton, 1951; Zaimis, 1976; Zaimis & Head 1976; Colquhoun, 1986). On the other hand, Thesleff (1955a,b) suggested that the neuromuscular block by succinylcholine (SCh) and decamethonium was

largely a result of desensitization of the acetylcholine (ACh) receptor, but not of depolarization *per se*, in frog, guinea-pig, rabbit, rat and cat muscles. Agonist-type muscle relaxants are also believed to block neuromuscular transmission by a dual mechanism, i.e., initially by depolarization and then, after a prolonged action, changing to a non-depolarizing block, presumably by desensitization of the receptor or by competitive inhibition (Zaimis, 1976). Since desensitization is an inactive closed state of the ACh-receptor channel occurring in the presence of an agonist, the outcome of desensitization is difficult to

Figure 1 Time-course of succinylcholine-induced block of indirect twitch responses of the mouse phrenic nerve diaphragm preparation: ($\square$) 20 μ M; ($\bullet$) 30 μ M; ($\circ$) 50 μ M. Points are mean values from 4 or more preparations; vertical bars show s.e.

discriminate from competitive blockade (Colquhoun, 1986). In addition to the postsynaptic effect, a presynaptic action has been reported for SCh (Edwards & Ikeda, 1962; Hubbard *et al.*, 1965; Standaert & Adams, 1965) but this mechanism is unlikely to be essential for the block of neuromuscular transmission (Colquhoun, 1986).

Acetylcholine receptors on the motor endplate, in addition to those subsynaptic junctional ones, have been shown to extend to the perijunctional area by as far as 25–50 μ m with decreasing densities (Salpeter *et al.*, 1988). The perijunctional receptor will be more easily accessible than the junctional one to exogenously applied agonists whereas the reverse is true for the neurally-released ACh. It has been shown that exogenous ACh preferentially affects extra-junctional receptors in chick biventer cervicis muscle with intact acetylcholinesterase and the response can be preferentially blocked by α -bungarotoxin (Chang & Tang, 1974; Chang & Su, 1975; Chang *et al.*, 1985). In this paper we show that α -bungarotoxin, but not tubocurarine, antagonized the SCh-induced depolarization more markedly than the depression of miniature endplate potential (m.e.p.p.) amplitude

while the neuromuscular block by SCh was enhanced. These results shed more light on the role of endplate depolarization and mode of action of SCh in neuromuscular blockade.

Methods

Twitch contractions

Hemidiaphragms of both right and left sides with the phrenic nerve attached were isolated from 20–25 g mice (ICR) of either sex after a blow on the head and bleeding. The organ bath contained 20 ml Tyrode solution (composition in mm: NaCl 137, KCl 2.8, CaCl_2 1.8, MgCl_2 1.1, NaH_2PO_4 0.33, NaHCO_3 11.9 and glucose 11.2) maintained at $37 \pm 0.5^\circ\text{C}$ and oxygenated with 95% O_2 + 5% CO_2 . The resting tension was 0.5 g. The nerve was stimulated with supramaximal pulses of 0.05 ms at 0.2 Hz in order to elicit indirect single twitch contractions. The diaphragm muscle was stimulated with a pair of long silver electrodes sandwiching the diaphragm at the margin of the rib end with submaximal pulses of 0.5 ms at 0.2 Hz to elicit direct twitch contractions. Contractions were recorded isometrically through a transducer (BG 25, Gould) coupled to a rectilinear physiorecorder (Gould 2200S).

Electrophysiological recordings

Experiments were carried out at $33 \pm 0.5^\circ\text{C}$ on the mouse phrenic nerve diaphragm preparation bathed in 12 ml Tyrode solution. Intracellular transmembrane potentials were recorded with glass capillary microelectrodes filled with 3 M KCl (5–10 M Ω) coupled to a high impedance d.c. amplifier (Grass P6). Microelectrodes were inserted into the endplate region of the diaphragm to record the resting transmembrane potential, spontaneous miniature endplate potentials (m.e.p.ps) and evoked endplate potentials (e.p.ps). M.e.p.ps or e.p.ps with rise times slower than 0.8 ms were rejected. The cut muscle preparation (Barstad & Lilleheil, 1968) of mouse diaphragm was equilibrated in the Tyrode solution for 2 h until the resting membrane potential declined to about -40 mV when the muscle fibre became incapable of generating action potentials, and evoked e.p.ps could be easily recorded. The amplitudes of m.e.p.ps and e.p.ps in the presence of SCh were corrected for the depolarization as previously described (Chang *et al.*, 1986).

Drugs

Succinylcholine chloride and tubocurarine chloride were obtained from Sigma Chemicals and α -

bungarotoxin was isolated from the crude venom of *Bungarus multicinctus* as previously described (Lee *et al.*, 1972).

Results

Succinylcholine-induced neuromuscular block and depolarization

In the isolated mouse phrenic nerve-diaphragm preparation, SCh did not depress the indirect twitch responses evoked by stimulation of the nerve at 0.2 Hz at concentrations lower than 10 μ M. At concentrations of 10–20 μ M, neuromuscular transmission was partially blocked progressively in 60 min. At concentrations of 30 μ M or higher, SCh caused a marked and rapid block within 5 min of application followed by a slight spontaneous recovery in the next 20–40 min before the second phase block took place (Figure 1). Figure 2 illustrates the relation between SCh concentrations and percentage neuromuscular blockade. The concentrations for 20% and 50% inhibition (IC_{20} , IC_{50}) at 40 min after SCh administration were 12 and 25 μ M, respectively. The mouse muscle is thus more than ten times as resistant to SCh as the cat muscle (Zaimis & Head, 1976).

The endplate area was rapidly and maximally depolarized from –80 mV to about –55 mV within 2–3 min after addition of SCh to the organ bath at concentrations 2 μ M and higher. The membrane potential was slightly restored to –60 mV in about 5 min and stayed at this level for more than 40 min (Figure 3). The median effective concentration (EC_{50}) of SCh for depolarization was about 0.6 μ M (Figure 2), being 20 times lower than the IC_{20} for the neuromuscular block. Whether SCh-induced depolarization results in a substantial inactivation of the sodium channel and thus leads to a neuromuscular block was studied by testing the effect of SCh on the twitch response to direct stimulation at the rib end of the diaphragm muscle. SCh showed no inhibitory effect on the direct twitch response at all (data not shown) even at concentrations up to 50 μ M which markedly disturbed the indirect twitch response.

Effects of succinylcholine on m.e.p.ps and e.p.ps

M.e.p.ps recorded from the uncut muscle preparations were reduced in amplitude dose-dependently by SCh with IC_{50} being about 0.76 μ M (Figure 2). This value is quite close to the SCh concentration which caused 50% maximal depolar-

Figure 2 Concentration-response curves of succinylcholine (SCh) for inducing neuromuscular blockade and endplate depolarization and for inhibiting m.e.p.ps and e.p.ps. M.e.p.ps (●), endplate depolarization (□) and neuromuscular block of indirect twitch responses (■) were measured in intact muscle preparations with resting membrane potential of about –80 mV. E.p.ps (○) were measured in cut muscles with resting membrane potential of about –40 mV. The effects of SCh were expressed as percentage taking complete inhibition as 100% except for depolarization where maximal depolarization by SCh (from –80 mV to –60 mV) was taken as 100%. Mean from 4 preparations or from >15 endplates; s.e. shown by vertical bars.

Figure 3 Time-course of endplate depolarization by succinylcholine (SCh) and effects of α -bungarotoxin and tubocurarine. SCh (10 μ M) was added at 0 min; (○) SCh only; (●) in the presence of tubocurarine (0.2 μ M); (□, ■) pretreated with 0.025 μ M α -bungarotoxin for 30 and 70 min, respectively. C denotes control. W denotes washout of SCh. Mean values from >20 endplates in 4 preparations; vertical bars show s.e.

Figure 4 Effects of succinylcholine (SCh) on the neuromuscular transmission partially blocked by tubocurarine (Tc) or α -bungarotoxin (α -BuTx). Left panels show the control twitch response. In (a) tubocurarine was added at $0.8 \mu\text{M}$ throughout, while in (b) the preparation was pretreated with α -bungarotoxin ($0.025 \mu\text{M}$) for 40 min and washed out. At (●) SCh ($15 \mu\text{M}$ in a, $10 \mu\text{M}$ in b) was added.

Figure 5 Effects of α -bungarotoxin and tubocurarine on the concentration-effect curves of succinylcholine (SCh) for neuromuscular block and endplate depolarization. Neuromuscular block by SCh in control (○), in the presence of $0.2 \mu\text{M}$ tubocurarine (Δ) and in preparations pretreated with $0.025 \mu\text{M}$ α -bungarotoxin (□) for 45 min and washout. Depolarization of endplate by SCh, as percentage of maximal depolarization (from -80 mV to -60 mV), in control (●), tubocurarine (▲) and α -bungarotoxin (■)-pretreated preparations. Mean from 4 preparations or >15 endplates; vertical bars show s.e.

Figure 6 Inhibition of m.e.p.s and succinylcholine (SCh)-induced depolarization by various concentrations of tubocurarine. The mouse diaphragm preparations were pretreated with tubocurarine for 30 min. The m.e.p.p. amplitudes (○) and SCh ($10 \mu\text{M}$)-induced depolarizations (●) in the presence of tubocurarine were expressed as percentage of control. Mean from >20 endplates in 3-4 preparations; vertical bars show s.e.

ization. On the other hand, e.p.s recorded from the cut muscle preparations were depressed by SCh at somewhat higher concentrations (Figure 2). To inhibit the e.p.p. amplitude by 50 and 80%, 1.2 and $6 \mu\text{M}$ SCh were needed, respectively. The latter concentration is close to the minimal effective concentration (IC_{20}) required to impair neuromuscular transmission.

Interaction with tubocurarine

Attempts to see whether tubocurarine (at various concentrations) could antagonize the neuromuscular blocking action of SCh in mice, as it does in cats (Zaimis & Head, 1976), were unsuccessful. It was found that SCh only temporarily antagonized the partial block caused by tubocurarine ($0.8 \mu\text{M}$) (Figure 4a). The neuromuscular block, however, was enhanced in the presence of tubocurarine. The IC_{50} was reduced from $25 \mu\text{M}$ to $18 \mu\text{M}$ in the presence of $0.2 \mu\text{M}$ tubocurarine (Figure 5). The spontaneous recovery following the initial block (cf. Figure 1) was no longer observed. On the other hand, the depolarization of the endplate caused by SCh was reduced dose-dependently by tubocurarine at 0.2 – $3 \mu\text{M}$ (Figure 6) and the concentration-depolarization

curve for SCh was shifted to the right in parallel by tubocurarine (Figure 5), suggesting the competitive nature of the antagonism between tubocurarine and SCh in depolarizing the endplate. The effect of tubocurarine on m.e.p.ps was compared with that on endplate depolarization caused by SCh at $10\text{ }\mu\text{M}$, the minimal concentration to impair neuromuscular transmission (Figure 6). The amplitude of m.e.p.p. was depressed by 50% by tubocurarine at $0.2\text{ }\mu\text{M}$, a concentration very close to its dissociation constant (Colquhoun, 1986), whereas $1.5\text{ }\mu\text{M}$ of tubocurarine was needed to attenuate by 50% the depolarization induced by SCh at $10\text{ }\mu\text{M}$.

Interaction with α -bungarotoxin

In preparations pretreated with α -bungarotoxin ($0.2\text{ }\mu\text{g ml}^{-1} = 0.025\text{ }\mu\text{M}$) for 45 min when the indirect twitch response was slightly inhibited, SCh ($10\text{ }\mu\text{M}$) caused synergistic augmentation of neuromuscular block without any initial transient reversal (Figure 4b) as seen in the tubocurarine-treated preparation. The median concentration of SCh to cause neuromuscular block was decreased from $25\text{ }\mu\text{M}$ in control preparations to $18\text{ }\mu\text{M}$ in the α -bungarotoxin-pretreated preparation (Figure 5). Figure 7 compares the effects of α -bungarotoxin after 15 to 60 min incu-

bation on the amplitude of m.e.p.ps and on the SCh ($10\text{ }\mu\text{M}$)-induced depolarization. Unlike tubocurarine, the SCh-induced depolarization was almost completely antagonized after 60 min incubation with α -bungarotoxin when the m.e.p.p. amplitude was depressed by only 50–60%. This result indicates that the SCh-depolarization was more vulnerable to the block by α -bungarotoxin than the response to neurally-released ACh. This situation is in contrast to that observed with tubocurarine. Also unlike tubocurarine, the depression of SCh-depolarization by α -bungarotoxin was not surmountable by increasing SCh concentrations (Figure 5).

Discussion

A comparison of the effective concentrations of SCh in causing endplate depolarization ($\text{EC}_{50} = 0.6\text{ }\mu\text{M}$; $\text{EC}_{80} = 1.2\text{ }\mu\text{M}$), in inhibiting m.e.p.ps ($\text{IC}_{50} = 0.76\text{ }\mu\text{M}$) and e.p.ps ($\text{IC}_{50} = 1.2\text{ }\mu\text{M}$; $\text{IC}_{80} = 6\text{ }\mu\text{M}$) and in causing a block of neuromuscular transmission (threshold concentration: $\text{IC}_{20} = 12\text{ }\mu\text{M}$) indicates that the cause for neuromuscular block induced by SCh in mice cannot be depolarization. This inference is further supported by the findings that the maximal depolarization reached only -60 mV and that the muscle action potential could still propagate through the endplate area during maximal depolarization indicating that the muscle sodium channel was not sufficiently inactivated to cause a neuromuscular blockade. These effects of SCh in mice are quite different from those in cats where the endplate can be depolarized to lower than -50 mV , which results in complete inactivation of the sodium channel and a depression of muscle contraction to direct stimulation (Zaimis & Head, 1976). Evidently, the difference in the magnitude of SCh-depolarization may result in different modes of neuromuscular blocking action by SCh and different potency in different species. Indeed, the mouse ACh receptors appear to have equal affinity for SCh as the cat receptors although they are much more resistant to its neuromuscular block. The cause of neuromuscular block by SCh in mice seems to be more probably the inhibition of e.p.ps as judged from the effective concentrations, considering a safety factor of 3 to 5 for murine diaphragm neuromuscular transmission (Chang *et al.*, 1975). The present data also show that m.e.p.ps were depressed slightly more than e.p.ps at low concentrations of SCh, implying that the quantal content of e.p.ps may be slightly increased. This is in line with the view that neurally-released ACh has a positive feedback on the release of neurotransmitter (Bowman *et al.*, 1986; Chang *et al.*, 1988).

Figure 7 Inhibition by α -bungarotoxin of m.e.p.ps and succinylcholine (SCh)-induced depolarization. The mouse diaphragm preparations were incubated with $0.025\text{ }\mu\text{M}$ α -bungarotoxin for various times and washed out. The m.e.p.p. amplitude (□) and SCh ($10\text{ }\mu\text{M}$)-induced depolarization (■) after toxin treatment were expressed as percentage of control. Mean from >20 endplates in 3–4 preparations; vertical bars show s.e.

Both tubocurarine and α -bungarotoxin are well known antagonists of the nicotinic ACh receptor of the motor endplate. They display qualitatively the same mode of neuromuscular blocking action except that tubocurarine, but not α -bungarotoxin, may additionally affect the open ACh channel (Katz & Miledi, 1978) and that the action of α -bungarotoxin is practically irreversible (Chang, 1979). The present study revealed that there are substantial differences in the interactions of α -bungarotoxin and tubocurarine with SCh. α -Bungarotoxin at $0.025 \mu\text{M}$ and tubocurarine at $0.2 \mu\text{M}$ were equipotent in inhibiting the amplitude of m.e.p.ps, which represents the response of junctional ACh receptor to neurally-released quanta of ACh, and in enhancing the neuromuscular block by SCh. On the other hand, the depolarizing response to SCh at the minimal concentration ($10 \mu\text{M}$) to impair neuromuscular transmission was more sensitive than m.e.p.ps to α -bungarotoxin and less sensitive to tubocurarine. The relationships between the inhibition of SCh-depolarization and the inhibition of m.e.p.p. by tubocurarine and by α -bungarotoxin, as summarized in Figure 8, are markedly different. These results suggest that SCh induces depolarization by acting on receptors different from those for m.e.p.ps. The difference in the actions of tubocurarine on the SCh-depolarization and m.e.p.ps may be explained on the basis of pharmacokinetics. Despite the fact that the binding of tubocurarine with the nicotinic receptor is reversible, practically no competition or displacement of tubocurarine from the nicotinic receptor can take place by the neurally-released ACh (cf. Colquhoun, 1986) because of the limited space in the synaptic cleft and the time available. Unlike neurally-released ACh, exogenous SCh has plenty of space and time available in competing with tubocurarine for inducing depolarization. The preferential block of SCh-depolarization by α -bungarotoxin may be due to a slow binding with the junctional receptor, since, at the toxin concentration used ($0.025 \mu\text{M}$), the free toxin molecule existing in the space of the synaptic cleft of one endplate (not more than $1 \mu\text{m}^3$) is less than 15 at equilibrium in the face of 10^7 receptors for binding. It is evident that the binding of α -bungarotoxin with junctional receptors would be

Figure 8 The relationships between the suppression of m.e.p.ps and succinylcholine (SCh)-induced depolarization by tubocurarine or by α -bungarotoxin. The preparations were either preincubated with α -bungarotoxin ($0.025 \mu\text{M}$) for various times and washed out (■) or treated with various concentrations of tubocurarine (●). Data from Figures 6 and 7.

much slowed compared to the binding with exposed perijunctional receptors, as previously shown in the chick muscle (Chang & Tang, 1974; Chang & Su, 1975).

Finally it may be inferred that SCh causes depolarization of the endplate by acting on perijunctional ACh receptors whereas the block of neuromuscular transmission in the mouse muscle by SCh is due to its effect on the junctional receptor. The latter effect is apparently not accompanied by sustained depolarization. The present results incidentally raise the possibility that the junctional ACh receptor of the endplate reacts with agonists differently from perijunctional ones in that the depolarization seems not to be maintained and is more prone to desensitization.

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