Succinylcholine-Induced Acceleration and Suppression of Electrically Evoked Acetylcholine Release from Mouse Phrenic Nerve-Hemidiaphragm Muscle Preparation

Ikuko Kimura, Mari Okazaki, Teruko Uwano, Shinjiro Kobayashi and Masayasu Kimura

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan

ABSTRACT—The effects of a depolarizing neuromuscular blocker on electrically evoked acetylcholine (ACh) release were studied using a mouse phrenic nerve-diaphragm muscle preparation preloaded with [3H]-choline, and the changes in muscle tension were recorded simultaneously. Succinylcholine at a low concentration (1 μM) enhanced evoked [3H]-ACh release, which tended to follow the increase in peak amplitude of tetanic tension; whereas at high concentrations (10 and 30 μM), it simultaneously reduced both release and tension. Decamethonium even at 10 and 30 μM had little effect on [3H]-ACh release despite producing a significantly greater reduction in tension compared with succinylcholine. (+)-Tubocurarine (5 μM) prevented the enhancing effect of [3H]-ACh release induced by 1 μM, but not the decreasing effect induced by 10 μM succinylcholine. These results suggest that succinylcholine induced acceleration at low concentrations due to a positive feedback mechanism through presynaptic nicotinic ACh receptors and the inhibition of ACh release at high concentration contributes in part to the neuromuscular blockade.

Presynaptic acetylcholine (ACh) receptors modulate ACh release from motor nerve terminals (1, 2). Electrophysiological and mechanical measurements suggest that (+)-tubocurarine may reduce the evoked release of ACh through blockade of a positive feedback mechanism mediated by presynaptic nicotinic ACh receptors (3, 4). The presynaptic mechanism of neuromuscular blockade induced by depolarizing blockers such as succinylcholine and decamethonium has not yet been elucidated in detail.

The aim of the present study was to determine whether succinylcholine has a direct effect on ACh release from the motor nerve endings of mouse diaphragm using the radioisotope method to count [3H]-efflux in the absence of cholinesterase inhibitors. The change in muscle tension was recorded simultaneously in the same preparation to clarify its relation to ACh release.

MATERIALS AND METHODS

Male ddY mice (7 - 10 weeks old, 31 - 41 g) were decapitated and bled. Either the right or left phrenic nerve-diaphragm muscle was removed and cut into strips about 10-mm wide together with the attached rib segment. The rib end of the preparation was pinned to rubber plates in a chamber, and the tendon was tied with a silk thread and connected to an
isometric transducer. The strip was suspended in a 2-ml chamber in Krebs solution gassed with a mixture of 95% O2 and 5% CO2 at 37°C. Krebs solution was composed of 113 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 25 mM NaHCO3, 1.2 mM KH2PO4, and 11.5 mM glucose.

**Measurement of [3H]-ACh in the perfusate**
A radioisotope method was used to measure the electrically evoked release of ACh without cholinesterase inhibitors (5–7). The above preparation was incubated for 60 min in 2 ml Krebs solution containing methyl [3H]-choline (370 kBq). To facilitate the uptake of [3H]-choline into the acetylcholine pool in motor nerve terminals, the preparation was stimulated for 40 min. For the next 20 min, the preparation was allowed to rest. The preparation was then washed with Krebs solution at a rate of 2 ml/min for 60 min to remove the excess [3H]-choline. The perfusion rate was then slowed to 1 ml/min. The phrenic nerve was stimulated four times. The 3-min stimulation periods started at 8 (S0), 32 (S1), 56 (S2) and 80 (S3) min after the washout period. Trains of 40 pulses (50 Hz, 0.2 msec duration, for 0.8 sec) were applied every 10 sec for 3 min by supramaximal square pulses (1-10 V) through a pair of platinum electrodes (7). Samples were collected every min from 9 min before the 32 min point (S1). The samples were collected into scintillation vials. Six milliliters of scintillation fluid (ACS-II, Amersham) was added to each one-min aliquot of perfusate. Radioactivity of the samples was measured in a scintillation beta spectrometer (Beckman, LS 3801). The stimulation-induced increase in [3H]-release was calculated by subtracting the mean of the basal release from the total release. Mean basal release was calculated from six data before and after a stimulation period. The tritium content of the tissue was measured after the perfusion experiment by placing the preparation into 2 ml of 0.4 N NaOH and counting a 50-μl aliquot.

**Radioimmunoassay for ACh in the perfusate**
The absolute amount of spontaneous and electrically evoked output of ACh from the preparation was determined in part by radioimmunoassay (8, 9) using ACh-antiserum. The experimental protocol was the same as that described above except for incubation with [3H]-choline. The preparation was treated with 10 mM methanesulfonyl fluoride, an irreversible acetylcholinesterase inhibitor, during incubation. The perfusate was collected continuously on ice. Then 630 μl of perfusate was incubated for 17 hr with 100 μl of diluted antiserum (1:300) in Tris-HCl buffer (0.1 M, pH 7.4) containing 0.4% bovine gamma-globulin, 0.05% diisopropylfluorophosphate, 10 μl of 10 mM acetic acid and 60 μl of tritiated ACh (4.8 pg, 5500 d.p.m.). The same volume of Krebs solution was used as a blank test. Antibody-bound ACh was separated from free tritiated ACh by the ammonium sulfate method and measured in a liquid scintillation counter. The presynaptic effect of succinylcholine cannot be determined by radioimmunoassay because of the high cross-reactivity of ACh-antiserum to succinylcholine.

**Peak amplitude of tetanic tension**
The isometric contractions were measured with a force displacement transducer (Nihon Kohden, SB1T) and recorded (Linea Recorder, Graphtec, WR3701). The resting tension was adjusted to 500 mg. The effects of drugs on the peak amplitude of tetanic tension were represented as T2/T1, where T was an averaged value of the 6th, 12th, and 18th peak amplitudes of tetanus for a 3-min stimulation period. T1 and T2 were produced by S1 and S2 stimulation periods, respectively.

**Statistics**
The unpaired t-test or one way analysis of variance was used. P < 0.05 was adopted as the level of significance.

**Drugs**
Methyl-[3H]-choline chloride (555 GBq/mmol), acetylmethyl-[3H]-choline chloride (2.78 TBq/mmol) (Amersham, Buckingham-
shire, England), succinylcholine chloride 2 H₂O, (+)-tubocurarine 3.5 H₂O (Nacalai Tesque, Kyoto, Japan), decamethonium bromide, gamma-globulin bovine Cohn fraction II, III (Sigma, St. Louis, MO, U.S.A.), acetylcholine chloride (Daiichi Seiyaku, Tokyo, Japan), and methanesulfonyl fluoride (Aldrich, Milwaukee, WI, U.S.A.) were used. The rabbit ACh-antiserum (NGC-10 880902, sensitivity of 10 pg/tube) was a gift from Prof. K. Kawashima (Department of Pharmacology, Kyoritsu College of Pharmacy, Japan).

RESULTS

The absolute value of resting release before S₁ was 2182 ± 383 Bq/(g of tissue) min⁻¹ for [³H]-efflux (n = 4). Electrically evoked [³H]-ACh release over the level of spontaneous output as determined by [³H]-efflux counting was compared with the amount of ACh determined by radioimmunoassay in Table 1. The S₂/S₁ ratio was not altered when the preparation was treated with 10 mM methanesulfonyl fluoride.

Radioimmunoassay showed that the absolute value of resting release before S₁ was 3.34 ± 0.25 ng/(g of tissue) min⁻¹ (n = 4). The absolute amount of ACh release was constant, and the S₂/S₁ value was not significantly different from 1.0. Since ACh-antiserum significantly cross-reacted with micromolar concentrations of succinylcholine (8), the effects of succinylcholine were investigated by [³H]-efflux counting.

Typical data showing the succinylcholine-induced effects on the evoked [³H] release are shown in Fig. 1. The S₂/S₁ ratio was 1.36 ± 0.15 at 1 μM succinylcholine (n = 4), demonstrating a significant enhancement of evoked ACh release. The ratio was 0.41 ± 0.06 at 10 μM succinylcholine (n = 4), indicating a significant inhibition of ACh release. The concentration-response curve for the ratios of evoked ACh release (S₂/S₁) was compared with that for the ratios of peak amplitude of tetanic tension (T₂/T₁) (Fig. 2). Both curves showed the following biphasic patterns: Low concentrations of succinylcholine enhanced and high concentrations reduced both ACh release and tetanic tension. Succinylcholine at 1 μM clearly enhanced the S₂/S₁ ratios, and 0.3 μM slightly enhanced the T₂/T₁ ratios. Both ratios were reduced significantly by 10 μM and 30 μM succinylcholine.

The concentration-response curves of decamethonium are shown in Fig. 3. Decamethonium at concentrations up to 10 μM did not have any significant effect on evoked ACh release despite the large decrease in the peak amplitude of tetanic tension at 10 μM. Even at a high concentration (30 μM), deca-

Table 1. Electrically evoked acetylcholine release from mouse phrenic nerve-diaphragm muscle preparation determined by [³H]-efflux counting compared with that determined by radioimmunoassay

|                | S₁          | S₂          | S₂/S₁       |
|----------------|-------------|-------------|-------------|
| [³H]-Efflux    |             |             |             |
| MSF non-treatment | 495 ± 103  | 377 ± 83    | 0.75 ± 0.03 |
| MSF treatment  | 320 ± 58    | 235 ± 83    | 0.70 ± 0.15 |
| Radioimmunoassay | 7.01 ± 1.90 | 6.29 ± 0.63 | 1.09 ± 0.19 |

Methanesulfonyl fluoride (10 mM, MSF) was administered during the 60 min-incubation period. Electrically evoked release (S₁, S₂) for 5 min starting after the 32nd and 56th min of collection was calculated by subtracting the spontaneous release from the total release. Each value is a mean ± S.E.M. (n = 4–5).
methonium reduced evoked ACh release by only 35% despite the 100% reduction in tension.

The effects of 1 and 10 μM succinylcholine on evoked ACh release in the presence and absence of 5 μM (+)-tubocurarine were compared (Fig. 4). (+)-Tubocurarine (5 μM) alone, completely inhibiting tension, had no effect on evoked ACh release, reduced the enhancement of ACh release induced by 1 μM succinylcholine, but did not antagonize the inhibition induced by 10 μM succinylcholine.

DISCUSSION

Presynaptic effects of succinylcholine have been proposed in addition to its postsynaptic effects. Succinylcholine in the presence of acetylcholinesterase inhibitor reduces the number of ACh quanta released from frog nerve-muscle preparation (10). Succinylcholine affects the motor nerve terminal in much lower doses than those which cause neuromuscular blockade on the soleus nerves and muscles of cats (11). The neuromuscular blockade caused by succinylcholine is dominated by the partial failure of rat phrenic nerve terminals to release ACh in stretched, partially depolarized muscles (12). All these suggestions are based on electrophysiological data.

ACh release from mouse phrenic nerve-hemidiaphragm preparations is measured directly in the presence of hemicholinium-3 to prevent the re-uptake of [3H]-choline (5-7). In the present study, both released ACh and muscle tension were measured simultaneously in response to electrical stimulation of the phrenic nerve without a cholinesterase inhibitor and without hemicholinium-3, which impairs ACh synthesis and produces a frequency-dependent fall-off in ACh output (4).

Our present study using the measurement of [3H]-ACh overflow indicated that the effect of succinylcholine is characterized by a two-phase pattern as follows: Succinylcholine enhanced ACh release and peak amplitude of tetanic tension at low concentrations, whereas it reduced them at high concentrations. Evoked ACh release was increased by a low, but 3 times higher concentration of succinylcholine than that at which tetanic tension tended to be increased. Increase in evoked ACh release was not accompanied with an increase in tension because evoked tetanic tension may have already been the maximal value. Therefore, the increase in tension induced by succinylcho-
Fig. 2. Concentration-response curves of succinylcholine (SuCh) for evoked [3H]-ACh release (closed symbols) and peak amplitude of tetanic tension (open symbols) in mouse phrenic nerve-diaphragm muscle preparation. Succinylcholine was administered in various concentrations 10 min before $S_2$. Stimulation was applied as described in Fig. 1. Each point represents the mean for relative ratios of ACh release ($S_2/S_1$) and tension ($T_2/T_1$), and vertical lines represent S.E.M. ($n = 4-6$). $T_1$ or $T_2$ is an averaged value of the 6th, 12th, and 18th peak amplitudes of tetanus produced by $S_1$ or $S_2$ stimulation period, respectively. Significant differences from the control (without drug) were analyzed by Student's t-test. **P < 0.01 and *P < 0.05.

Fig. 3. Concentration-response curves of decamethonium ($C_{10}$) for evoked [3H]-ACh release (closed symbols) and peak tetanic tension (open symbols). Decamethonium was administered in various concentrations 10 min before $S_2$. Stimulation was applied as described in Fig. 2. Points and vertical lines are the same as in Fig. 2 ($n = 4-6$). Significant differences from the control (without drug) as determined by Student's t-test. **P < 0.01.
line may not be due to the increase in evoked ACh release. Evoked ACh release was decreased by high concentrations of succinylcholine, which were accompanied by simultaneous suppression of the peak amplitude of tetanic tension to the same extent. Electrophysiological data suggest that the cause of the neuromuscular block by succinylcholine is a postsynaptic inhibitory effect, rather than a presynaptic one (13). However, our results suggest that the mechanism of neuromuscular blockade induced by succinylcholine is in part due to presynaptic suppression.

The two depolarizing neuromuscular blockers were quite different in their presynaptic actions. In contrast to succinylcholine, decamethonium hardly affected the motor nerve terminal. The neuromuscular block induced by decamethonium is, therefore, attributable to postsynaptic inhibition alone. Succinylcholine had a greater effect on the motor nerve terminal than decamethonium.

Presynaptic nicotinic receptors facilitate ACh release by a positive feedback mechanism, the evidence for which has been provided by electrophysiological (14), pharmacological (15, 3, 4) and radioisotope methods (16 -18). Succinylcholine at low concentration may promote the positive feedback mechanism by presynaptic nicotinic ACh receptors, the enhancing effect of succinylcholine on evoked ACh release was completely inhibited by (+)-tubocurarine. (+)-Tubocurarine may suppress it by reacting with presynaptic n-ACh receptors. No inhibitory effect of (+)-tubocurarine on ACh release in the presence of acetylcholinesterase inhibitor has been demonstrated (19, 20): the desensitization of presynaptic nicotinic receptors may develop rapidly in the presence of high concentrations of agonists or cholinesterase inhibitors (16). Presynaptic nicotinic receptors are more sensitive to desensitization than postsynaptic ones (21). The inhibitory action of succinylcholine was not antagonized by (+)-tubocurarine; thus the high concentrations of succinylcholine may have desensitized
presynaptic nicotinic ACh receptors.

In conclusion, succinylcholine-induced acceleration of ACh release at low concentration is due to positive-feedback mechanisms involving presynaptic nicotinic ACh receptors and the inhibition of ACh release at high concentration may contribute to the neuromuscular blockade.

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