Streptozocin-Diabetes Modifies Acetylcholine Release from Mouse Phrenic Nerve Terminal and Presynaptic Sensitivity to Succinylcholine

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ABSTRACT—Acetylcholine (ACh) release from the motor nerve terminal in the streptozocin-induced diabetic state was studied in mouse phrenic nerve-diaphragm muscle preparations. Electrically evoked release of ^{3}H-ACh from the preparation preloaded with ^{3}H-choline was measured during two consecutive periods of stimulation (S_1 and S_2). In diabetic mice, the amount of ^{3}H-ACh release during S_2 was decreased, and the evoked ACh release declined more steeply with successive stimulation periods than in normal ddY mice. The decrease in release was restored when the presynaptic autoreceptors were stimulated by accumulating ACh under the irreversible inhibition of junctional cholinesterase by methanesulfonyl fluoride. This effect was abolished by the administration of (+)-tubocurarine (5 μM). In diabetic mice, the biphasic (acceleration and suppression) effect by succinylcholine on evoked ACh release was caused at 3- to 10-fold lower concentrations than in normal mice. The degree of enhancement of resting ^{3}H-overflow by succinylcholine (10 and 30 μM) was greater in the diabetic state. These results indicated that in the diabetic state, the decrease in evoked ACh release interferes with its presynaptic action on inducing further release (positive feedback modulation) via the presynaptic nicotinic ACh receptor (n-AChR). The presynaptic hypersensitivity to succinylcholine may be due to the augmentation of presynaptic n-AChR sensitivity caused by the reduction of evoked ACh release in the diabetic state.

Keywords: Acetylcholine release (evoked, resting), Succinylcholine, Nicotinic acetylcholine receptor (presynaptic), Phrenic nerve-diaphragm muscle (mouse), Streptozocin-diabetes

Diabetic neuropathy, a disorder of peripheral nerves particularly, is one of the most common complications of diabetes mellitus. Nerve conduction velocity is reduced in diabetic peripheral neurons, and pathogenetic mechanisms responsible for the impairment of axons and Schwann cells have been investigated (1). Few studies have examined whether the diabetic state affects transmitter release from motor nerve terminals.

Electrophysiological and mechanical studies showed that diabetes causes functional changes in neuromuscular transmission (2, 3). Succinylcholine is hypersensitive at the neuromuscular junction in the diabetic state (4, 5), and the hypersensitivity is mainly attributed to the diabetic skeletal muscle cells (6, 7). The change in presynaptic sensitivity to succinylcholine in diabetes has not yet been studied with regard to the abnormalities of presynaptic function associated with diabetic neuropathy. We have previously reported that succinylcholine affected nerve-

evoked acetylcholine (ACh) release from the phrenic nerve terminal of normal ddY mice through presynaptic nicotinic receptors (8). The present study was designed to evaluate the change of ACh release and the sensitivity of nerve terminal to succinylcholine in the streptozocin (STZ)-induced diabetic state by the ^{3}H-efflux counting method and radioimmunoassay using mouse phrenic nerve-hemidiaphragm muscle preparations.

MATERIALS AND METHODS

Male ddY mice were used after 4−5 weeks of diabetes (8- to 9-week-old, 27.5−36.5 g body weight, blood glucose level: 19.4−37.0 mM) induced by the administration of 150 mg/kg STZ, which was dissolved in 0.9% NaCl and injected into the tail veins of the mice. Normal male ddY mice (7- to 10-week-old, 31−41 g body weight, blood glucose level: 5.8−7.3 mM) were used as the controls. Blood

glucose levels were measured by the glucose oxidase method with a glucose analyzer (Type II, Beckman, Fullerton, CA, USA).

The mouse was killed by decapitation. The right or left phrenic nerve-diaphragm muscle was isolated as previously reported (8) and then suspended in a 2-ml chamber containing Krebs' solution gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C. Krebs solution was composed of 113 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 11.5 mM glucose.

Measurement of ^3H-ACh in the perfusate

The above preparation was incubated for 60 min in 2-ml Krebs solution containing methyl-^3H-choline. The preparation was stimulated for 40 min with trains of pulses, as described below, and allowed to rest for the next 20 min. After the incubation period, the preparation was then washed with Krebs solution at a rate of 2 ml/min for 60 min to remove excess ^3H-choline. The perfusion rate was then slowed to 1 ml/min, and the phrenic nerve was stimulated electrically for 3 min, four times starting at 8 (S_0), 32 (S_1), 56 (S_2) and 80 (S_3) min after the end of the washout period. Trains of 40 pulses (50 Hz, 0.2 msec duration, for 0.8 sec) were applied to the phrenic nerve every 10 sec for 3 min by supramaximal square pulses through a pair of platinum electrodes (1 – 10 V). Samples were collected every min from 9 min before the 32-min point (S_1).

Radioactivity of the samples was measured by a scintillation beta spectrometer (LS 3801, Beckman). The stimulation-induced increase in tritium 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 effects of drugs on the evoked release of ACh were detected by changes in the S_2/S_1 ratios.

The effect of succinylcholine on basal tritium efflux was examined by calculating the ratio of tritium radioactivity of 10-min samples collected after the addition of succinylcholine (B_2) to that of samples collected before the addition (B_1). In the control experiments, the B_2/B_1 ratio was also obtained in the absence of succinylcholine.

Separation of ^3H-choline and ^3H-ACh

The ^3H-choline and ^3H-ACh fractions were separated in each perfusate by a modified choline kinase method (9). Each 500-μl aliquot of perfusate was treated for 15 min with 20 μl of choline kinase (10 mU/ml) in the presence of 125 μl of sodium phosphate buffer (pH 8.0), 50 μl of 10 mM ATP and $MgCl_2$. Choline kinase converts ^3H-choline to ^3H-phosphorylcholine. The mixture of ^3H-ACh and ^3H-phosphorylcholine was then separated by

stirring with 1 ml 3-heptanone containing 30 mM tetraphenylborate. ^3H-Phosphorylcholine remained in the aqueous phase under these conditions, but ^3H-ACh was distributed quantitatively into the upper 3-heptanone phase. A portion of the 3-heptanone phase was transferred to a tube containing 800 μl in 1 N HCl and mixed, and then the radioactivity of tritium in the HCl phase was measured as ^3H-ACh.

Radioimmunoassay for ACh in the perfusate

The absolute amount of spontaneous and electrically evoked ACh release from the preparation was determined in part by radioimmunoassay (8, 10, 11) with ACh-antiserum. The experimental protocol was the same as that described above except for the incubation with ^3H-choline. The preparation was treated with 10 mM methanesulfonyl fluoride (MSF), an irreversible cholinesterase inhibitor, during the incubation period to prevent hydrolysis of ACh. 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 ^3H-ACh (4.8 pg, 5500 dpm). The same volume of Krebs solution was used for a blank test. After removing free ^3H-ACh by the ammonium sulfate method, antibody-bound ACh was measured in the liquid scintillation beta counter. ACh-antiserum cannot be used to determine the presynaptic effect of succinylcholine because it shows high cross-reactivity to succinylcholine.

Peak amplitude of tetanic tension

The isometric contractions were measured with a force displacement transducer (SB1T, Nihon Kohden, Tokyo) and recorded (Linea Recorder, WR3701, Graphtec, Tokyo). The resting tension of 500 mg was loaded in each preparation. The effects of drugs on the peak amplitude of tetanic tension were represented as T_2/T_1, where T was an averaged value of the 6th, 12th and 18th peak amplitude of tetanic tension for a 3-min stimulation period. T_1 and T_2 were produced by the S_1 and S_2 stimulation periods, respectively.

Statistics

Statistical significances of the differences were analyzed by the unpaired Student's t-test. $P < 0.05$ was adopted as the level of significance.

Drugs

Methyl-^3H-choline chloride (555 GBq/mmol), acetyl-methyl-^3H-choline chloride (2.78 TBq/mmol) (Amersham, Buckinghamshire, England), succinylcholine chloride $2H_2O$, gamma-globulin bovine Cohn fraction II, III,

streptozocin, choline kinase (Sigma, St. Louis, MO, USA), acetylcholine chloride (Daiichi Seiyaku, Tokyo) and methanesulfonyl fluoride, 3-heptanone (Aldrich, Milwaukee, WI, USA) and sodium tetraphenylborate (Wako, Osaka) were used. 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

Decrease in evoked ACh release by successive electrical stimulation periods in the diabetic state

Electrically evoked ^{3}H-ACh release over the level of spontaneous output from the phrenic nerve-diaphragm muscle preparation of STZ diabetic mice was determined by measuring the overflow of tritium (Table 1). The release of radioactivity at rest before S_1 was 3212.4±248.1 dpm/min (n=8) in diabetic mice, which was not different from 3520±232.3 dpm/min (n=10) in normal mice. In the ^{3}H-efflux counting method, the amount of evoked

^{3}H-ACh release declined with successive stimulation periods. The time-dependent decline of release is due to the diminution of preloaded ^{3}H-choline. The amount of ^{3}H-ACh release evoked by the S_2-stimulation period was significantly reduced in diabetic mice, whereas that evoked by S_1 was unchanged, and the S_2/S_1 ratio was smaller in diabetic mice than in normal ones. The release from preparations pretreated with MSF, an irreversible cholinesterase inhibitor, was the same in the diabetic state as in the normal state. (+)-Tubocurarine at 5 μM, which had no effect on ACh release, abolished the MSF treatment-induced enhancement of ^{3}H-ACh release evoked by S_2 in the diabetic state.

The radioimmunoassay using MSF also showed that the absolute values of evoked ACh release were constant through two stimulation periods both in normal and diabetic mice and tended to be increased in the diabetic neuromuscular junction (Table 2). Resting ACh release from diabetic preparations was about 2.5-fold larger than that from normal ones.

Table 1. Electrically evoked acetylcholine release from normal and streptozocin (STZ)-diabetic mouse phrenic nerve-diaphragm muscle preparation determined by ^{3}H-efflux counting method

	S_1	S_2	S_2/S_1	5 μM d-TC S_2/S_1
Normal ddY	dpm/min			
	671±51	475± 50	0.70±0.02	0.64±0.07
MSF treatment	484±89	391±120	0.70±0.15	0.62±0.07
STZ 4-5-week-elapsed		**	**	
	479±92	227± 48	0.48±0.04	0.36±0.11
MSF treatment	531±61	390± 37	0.74±0.01 **	0.48±0.03††

Methanesulfonyl fluoride (10 mM, MSF) was administered during the 60-min incubation period. Electrically evoked release (S_1, S_2) for 3 min starting after the 32nd and 56th min of collection was calculated by subtracting spontaneous release from the total release. (+)-Tubocurarine (5 μM, d-TC) was administered 10 min before S_2. Values are means±S.E. (n=3-6). The significance of differences was analyzed by Student's t-test. **P<0.01. ††P<0.01, significantly different from the value without d-TC.

Table 2. Resting and electrically evoked acetylcholine release from normal and streptozocin (STZ)-diabetic mouse phrenic nerve-diaphragm muscle preparation determined by radioimmunoassay

	Resting release B	Evoked release S_1	S_2	S_2/S_1
	pg/min			
Normal ddY	67.8± 6.0 *	104.9±19.6	133.6±12.8	1.37±0.15
STZ 4-5-week-elapsed	177.8±33.8	175.0±34.4	188.5±27.7	1.13±0.16

Methanesulfonyl fluoride was administered during the 60-min incubation period. Electrically evoked release (S_1, S_2) for 3 min starting after the 32nd and 56th min of collection was calculated by subtracting the resting release (B, the mean of basal release) from the total release. Values are means±S.E. (n=3-5). The significance of difference was analyzed by Student's t-test. *P<0.05.

The effect of succinylcholine on evoked ACh release and peak tetanic tension in the diabetic state

The concentration-response curves of succinylcholine for the ratios of evoked ACh release (S_2/S_1) and peak amplitude of tetanic tension (T_2/T_1) shows biphasic patterns (Fig. 1), like in the normal state as previously reported (8). Succinylcholine ($0.1 - 10$ μM) enhances ACh release and peak tetanic tension at low concentrations, but reduces them at high concentrations. The IC_{50} (95% confidence limit) for S_2/S_1 and T_2/T_1 were 1.9 μM ($1.1 - 3.3$) and 3.3 μM ($2.3 - 4.7$), respectively.

Effects of succinylcholine on basal tritium overflow in the diabetic state

B_2/B_1 (the ratio of basal release) without succinylcholine was 0.88 ± 0.01 in the normal mice ($n = 10$) and 0.88 ± 0.02 in the diabetic mice ($n = 8$). Figure 2 shows typical data of the increasing effect of succinylcholine (10 μM) on basal tritium release. Succinylcholine increased the B_2/B_1 ratio in a concentration-dependent manner in both states (Fig. 3). The effect caused by 10 and 30 μM succinylcholine was significantly greater in diabetic mice than in normal ones.

The spontaneous release of tritium enhanced by succinylcholine was separated into ^{3}H-choline and ^{3}H-ACh by the modified choline kinase method. Both the overflow of ^{3}H-choline and ^{3}H-ACh were increased spontaneously by succinylcholine, whereas only ^{3}H-ACh release was evoked by electrical nerve stimulation (Fig. 4). The ratio of ^{3}H-ACh release to total tritium release spontaneously

induced by 30 μM succinylcholine was compared between normal and diabetic preparations. The two ratios (normal: 0.53 ± 0.01, and diabetic: 0.45 ± 0.04, $n = 3$) were not significantly different from each other.
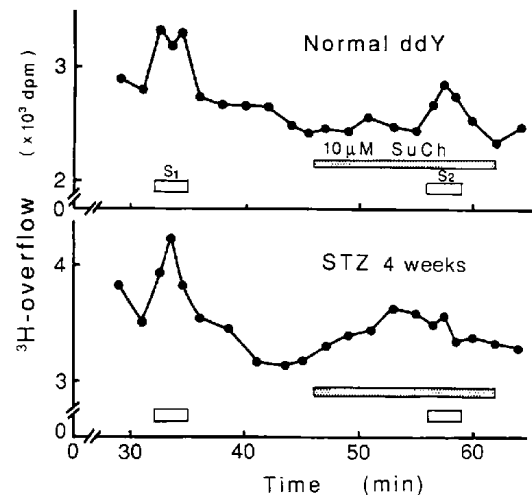

Fig. 2. Typical data showing the effects of succinylcholine (SuCh) (10 μM) on the release of tritium radioactivity from normal (upper) and streptozocin (STZ)-induced diabetic (lower) mouse hemidiaphragm preparations. Periods of stimulation (50 Hz, trains of 40 shocks) were applied every 10 sec as indicated by the open columns. The shaded columns indicate the presence of succinylcholine. The horizontal axis indicates the time after the end of the washout period.
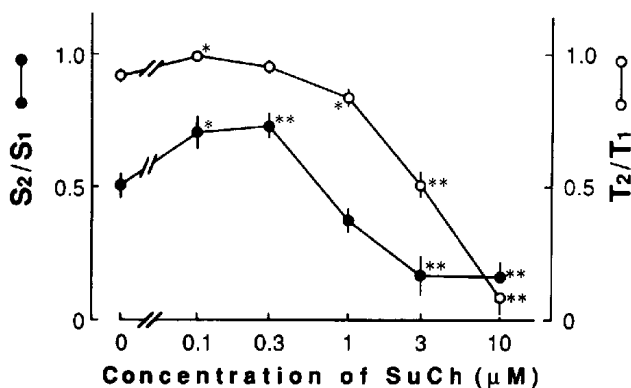

Fig. 1. Concentration-response curves of succinylcholine (SuCh) for evoked ^{3}H-ACh release (closed symbols) and peak amplitude of tetanic tension (open symbols) in streptozocin (STZ)-induced diabetic mouse phrenic nerve-muscle preparations. Succinylcholine was administered at various concentrations 10 min before S_2. Each point represents the mean value for relative ratios of ACh release (S_2/S_1) and tension (T_2/T_1), and vertical lines represent S.E. ($n = 4 - 6$). The significance of differences from controls (without drug) was analyzed by Student's t-test. **$P < 0.01$ and *$P < 0.05$.
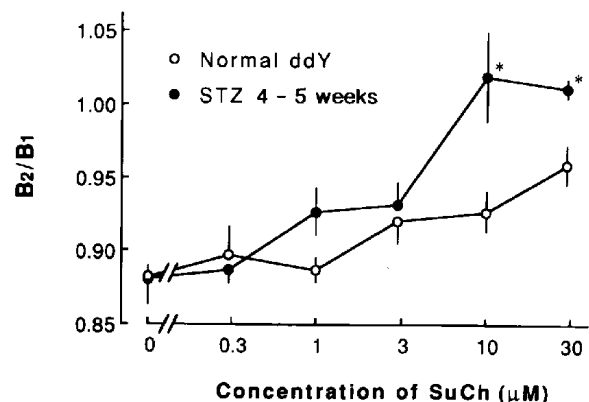

Fig. 3. Effect of succinylcholine (SuCh) on basal ^{3}H-overflow (B_2/B_1 ratio) from normal (open symbols) and streptozocin (STZ)-induced diabetic (closed symbols) mouse hemidiaphragm preparations. B_1 and B_2 are the total values of 10 min-samples collected before and after the addition of succinylcholine, respectively. Each point represents the mean value ($n = 3 - 10$) and vertical lines indicate S.E. The significance of differences from controls (normal ddY mice) was analyzed by Student's t-test. *$P < 0.05$.
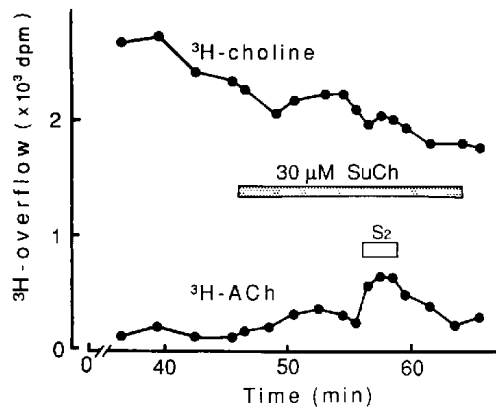
Fig. 4. Typical data showing the effects of succinylcholine (SuCh, 30 μM) on the overflow of ^{3}H-choline (upper line) and ^{3}H-acetylcholine (ACh) (lower line). Radioactive choline and ACh in the perfusate were separated by the modified choline kinase method. Periods of stimulation (50 Hz, trains of 40 shocks) were applied every 10 sec as indicated by the open column. The shaded column indicates the presence of succinylcholine. The horizontal axis indicates the time after the end of the washout period.

DISCUSSION

The abnormalities of neuromuscular transmission and the changes in sensitivity of the neuromuscular junction to various muscle relaxants in the diabetic state have been investigated. Schofield et al. (12) have demonstrated that phrenic nerve-diaphragm muscle preparations from alloxan-diabetic rats show reduced sensitivity to the neuromuscular blocking effect of (+)-tubocurarine, but no change is observed with decamethonium, a depolarizing neuromuscular blocker. However, our data do not agree with their data, because we (5) have observed that in situ sciatic nerve-gastrocnemius muscle preparations of alloxan-diabetic mice and genetically diabetic male KK-CA^y mice are hypersensitive to succinylcholine in the twitch-blocking effect. Furthermore, succinylcholine reduces the amplitude of ACh potential at lower concentrations in diaphragm muscles from diabetic KK-CA^y than normal mice. These data suggest that the postsynapse or the muscle cells themselves are hypersensitive to succinylcholine. Our present study demonstrates that diabetes rendered the presynapse also more sensitive to succinylcholine. We previously reported that succinylcholine (0.3–30 μM) shows a biphasic effect to both evoked ACh release and peak tetanic tension: acceleration at low concentrations and suppression at high concentrations (IC$_{50}$ of S$_2$/S$_1$: 14.6 μM, T$_2$/T$_1$: 7.1 μM) (8). The diabetic state shifted the curves of both release and tension 3- to 10-fold to the left and diminished the extent of enhancement of evoked ACh release at the low concentration of succinylcholine (Fig. 1). Succinylcholine induced the inhibition of evoked

ACh release 7.7-fold more potently in diabetic mice than in normal ones. Since succinylcholine affects ACh release through presynaptic nicotinic ACh receptor (n-AChR) (8), this result suggests that the susceptibility to succinylcholine of presynaptic n-AChR is increased in the diabetic state.

Succinylcholine increased basal tritium overflow in the resting state (Figs. 2 and 3). It is reported that carbamylcholine causes an increase in the frequency of the miniature end-plate potential along with a postsynaptic block in rat hemidiaphragm preparation (13). The frequency is augmented by depolarization of the nerve terminal (13, 14). Since motor nerves have nicotinic receptors that mediate membrane depolarization (15, 16), succinylcholine may depolarize the nerve terminals through these receptors and increase basal tritium overflow. In diabetic mice, the absolute amount of resting ACh release measured by radioimmunoassay (Table 2) and the extent of enhancement of resting tritium release induced by high concentrations of succinylcholine (Fig. 3) were greater than in normal mice. An electrophysiological study has shown that ACh is released mainly in a nonquantal manner at rest, whereas quantal release also occurred spontaneously, generating miniature end-plate potential (17). The nonquantal ACh leakage from the motor nerve terminal is increased by ouabain, a Na$^+$,K$^+$-ATPase inhibitor (18). The activity of Na$^+$,K$^+$-ATPase is reduced in diabetic neurons (19, 20), which may cause an increase of ACh release in the resting state. The hyperresponse to succinylcholine in resting release may be due to the depolarization of diabetic nerve terminals caused by reduction of the enzyme activity and/or increase of nonquantal ACh release. Succinylcholine increased the overflow of ^{3}H-choline in addition to that of ^{3}H-ACh (Fig. 4), whereas electrical nerve stimulation or high K$^+$ solution (21) causes selective liberation of ^{3}H-ACh but not ^{3}H-choline. Diabetes enhanced succinylcholine-induced spontaneous overflow of both ACh and choline to the similar extent. The source and mechanism of choline liberation remains uncertain.

The mouse hemidiaphragm preparations synthesize ^{3}H-ACh from ^{3}H-choline taken up during the incubation period. The newly synthesized ^{3}H-ACh is selectively released from the phrenic nerve terminal by electrical nerve stimulation. In diabetic mice, the amount of tritium release was reduced by repeated stimulation periods, whereas that during S$_1$ was unchanged (Table 1). This result suggests that ACh release induced by tetanic nerve stimulation is easily decreased in diabetic nerves. Low concentrations of succinylcholine enhanced evoked ^{3}H-ACh release to smaller extent in the diabetic state (Fig. 1) than in the normal state (8). Therefore, the number of releasable vesicles may be reduced in the diabetic nerve terminal. The reduced amount of ACh release caused by suc-

cessive stimulation periods in the diabetic state was restored by pretreatment with MSF. Inhibition of tissue cholinesterase raised the concentration of endogeneously released ACh in the synaptic cleft, and then intensified the effect of ACh on presynaptic autoreceptors. Evidence has been obtained that ACh enhances its own release from motor nerve terminals through presynaptic nicotinic receptors during high frequency nerve stimulation (22–24). The nicotinic positive feedback mechanism is physiologically predominant over other feedback modulations to retain ACh release during high frequency stimulation (25). In diabetic motor nerve terminals, the nicotinic positive feedback mechanism could not operate effectively because of the reduction of evoked ACh release. (+)-Tubocurarine completely inhibited the effect of MSF treatment, supporting the above explanation (Table 1). The release was unchanged with MSF treatment in normal mice; the susceptibility of facilitatory nicotinic autoreceptors is relatively higher than other inhibitory receptors in diabetic motor nerve terminals. In the neuromuscular junction, nerve impulse-evoked ACh release from motor nerve terminals influence muscle activity. Denervation of adult skeletal muscle increase the sensitivity to ACh in extrajunctional regions of the muscle fiber and increases n-AChR mRNA levels (26, 27). The reduction in evoked ACh release induced by the diabetic state may lead to an increase in the number of n-AChR in nerve terminals.

In conclusion, the diabetic state reduced evoked ACh release and increased resting release. The evoked/resting release ratio was lower in the diabetic state than in the normal state. In the diabetic state, the sensitivity of the motor nerve terminal containing presynaptic n-AChR to succinylcholine was increased. The hypersensitivity of the presynaptic response to succinylcholine may be due to augmentation of the sensitivity of presynaptic n-AChR in the diabetic state.

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