Aconitine-Induced Increase and Decrease of Acetylcholine Release in the Mouse Phrenic Nerve-Hemidiaphragm Muscle Preparation

Mari Okazaki, Ikuko Kimura* and Masayasu Kimura

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930–01, Japan

ABSTRACT—The effect of aconitine on acetylcholine (ACh) release from motor nerve terminals in the mouse phrenic nerve-diaphragm muscle preparation was studied by a radioisotope method. Both electrical stimulation-evoked release and spontaneous release of ^{3}H-ACh from the preparation preloaded with ^{3}H-choline were measured. The change in the muscle tension was simultaneously recorded in the same preparation. Aconitine (0.1 μM) increased electrically evoked ^{3}H-ACh release, while at higher concentrations (0.3–3 μM) it decreased the evoked release and muscle tension. High concentrations of aconitine (3–30 μM) caused a concentration-dependent increase in spontaneous ^{3}H-ACh release. All these effects were suppressed by tetrodotoxin. The aconitine-induced spontaneous release consisted of two different components: a Ca^{2+}-dependent phasic release that was inactivated within a few minutes and a Ca^{2+}-independent, long lasting release at a low level. The depression of the Ca^{2-}-dependent quantal release seems attributable to the decline of Ca^{2+} influx into the nerve rather than inactivation of sodium channels. We conclude that aconitine increases and then decreases electrical stimulation-evoked ACh release from the motor nerve through prolonged activation of sodium channels. Further activation of the channels enhances spontaneous release and the subsequent complete inactivation of the quantal release may be due to block of Ca^{2+} influx.

Keywords: Aconitine, Acetylcholine release (evoked, spontaneous), Tetrodotoxin, Phrenic nerve-diaphragm muscle (mouse)

Aconitine is one of the alkaloid neurotoxins that are known to modify the properties of voltage-sensitive sodium channels in excitable cell membranes (1). This alkaloid binds to a second receptor site associated with the channels (2). The alteration of the gating function of sodium channels in neuronal cell membrane induced by aconitine has been investigated electrophysiologically in detail (3). The agent eliminates the inactivation of normal sodium channels and forms non-inactivating channels that are fully activated at the resting membrane potential (3, 4). As a result of these effects, aconitine produces an irreversible depolarization of cell membranes (3, 5). However, how the aconitine-induced persistent activation of sodium channels in nerves affects transmitter release is not clear. Aconitine increases the frequency of miniature end-plate potential in rat neuromuscular junction, which is inhibited by tetrodotoxin (TTX) (6). On the other hand, aconitine reduces electrically evoked quantal acetyl-

choline (ACh) release via inhibition of nerve action potential (5), and it markedly inhibits spontaneous non-quantal release measured by the electrophysiological method (7) in the phrenic nerve-diaphragm muscle of mice. In this study, we investigated the effects of aconitine on both electrical stimulation-evoked release and spontaneous release of ACh by directly measuring the efflux of ^{3}H-ACh from the mouse phrenic nerve-hemidiaphragm muscle preparation which was preloaded with ^{3}H-choline.

MATERIALS AND METHODS

Male ddY mice (7- to 9-week-old, 30–41 g) were decapitated and bled. The right phrenic nerve-diaphragm muscle was isolated and cut into a strip 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 2 ml Krebs solution gassed with a mixture of 95% O_2

---

* To whom correspondence should be addressed.

and 5% CO_2 at 37℃. 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 radioactivity for ^3H-ACh in the perfusate

A radioisotope method was used to measure the release of ACh without cholinesterase inhibitors (8–10). 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 (50 Hz for 0.8 sec administered every 10 sec, 0.2-msec duration, 2.5 V) for 40 min. For the next 20 min, the preparation was allowed to rest. In the experiments to measure the release of radioactive compounds (^3H-ACh and ^3H-choline), the preparation was treated with 10 mM methanesulfonyl fluoride, an irreversible cholinesterase inhibitor, during the incubation period in order to prevent hydrolysis of released ^3H-ACh. The preparation was then washed with Krebs solution at a rate of 2 ml/min for 60 min to remove the excess ^3H-choline. Subsequently, the perfusion rate was slowed to 1 ml/min. The periods of the above electrical stimulation for 3 min were started at 8 (S_0) and 32 (S_1) min after the washout period. Additionally, S_2, which started 21 min after the end of S_1, was applied to investigate the effect of aconitine on the electrically evoked ACh release. Aconitine and TTX were administered to the perfusing solution 11 and 6 min after S_1 (10 and 15 min before S_2), respectively. In the experiments to study the effect of Ca^{2+} deprivation, the Ca^{2+}-free solution containing 1 mM EGTA was perfused 10 min before the addition of aconitine. Samples were collected every min from 6 min before the 32-min point (S_1). Six milliliters of scintillation fluid (ACS-II, Amersham) was added to each 1-min aliquot of perfusate. The radioactivity of the samples was measured in a scintillation beta spectrometer (LS 3801; Beckman, Fullerton, CA, USA).

The stimulation-induced increase in ^3H-release was calculated by subtracting the mean of the basal release from that of the evoked release. The mean basal release was calculated by the values of six fractions before and after a stimulation period. The effects of drugs on the evoked release of ACh were determined by changes in the S_2/S_1 ratios. The aconitine-induced increase in spontaneous release was estimated from the difference between the expected basal tritium outflow and the actually measured one in the presence of aconitine. The expected outflow-curve for basal release was calculated as follows: 1) The curves of control experiments (n=4) were estimated by fitting the data to the corresponding one-exponential curves

by the method of least squares, since spontaneous outflow is assumed to follow first-order kinetics (10). 2) The values of the slope and intercept for the expected outflow-curve of each experiment were calculated from the control curves using the values of tritium radioactivity of the 5-min period just before the addition of aconitine.

Peak amplitude of tetanic tension

The isometric contraction of the diaphragm muscle was simultaneously measured with a force displacement transducer (SB1T; Nihon Kohden, Tokyo) and recorded (Linea Recorder, WR3701; Graphtec, Tokyo). The resting tension was adjusted to 500 mg. The effect of aconitine on the peak amplitude of the tetanic tension was represented as T_2/T_1, where T was an averaged value of the 6th, 12th and 18th peak amplitudes of tetanus for a 3 min-stimulation period. T_1 and T_2 were produced by S_1 and S_2 stimulation periods, respectively.

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

^3H-choline and ^3H-ACh in each perfusate were separated into individual fractions by a modified choline kinase method (9, 11). A 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 200 mM sodium phosphate buffer (pH 8.0), 50 μl of 10 mM ATP and 10 mM $MgCl_2$. 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, whereas ^3H-ACh was distributed quantitatively into the upper 3-heptanone phase which was transferred to a tube containing 800 μl 1 N HCl and mixed. Then the radioactivity of tritium in the HCl phase was measured as ^3H-ACh.

Statistics

The statistical significance of the differences between the treated groups and the corresponding control was determined by one way ANOVA followed by Scheffe's test or two way ANOVA followed by a studentized range test. $P < 0.05$ was adopted as the level of significance.

Drugs

Methyl-[^3H]-choline chloride (555 GBq/mmol; Amersham, Buckinghamshire, England); methanesulfonyl fluoride, 3-heptanone (Aldrich, Milwaukee, WI, USA); O,O'-bis (2-aminoethyl) ethyleneglycol-N,N,N',N'-tetraacetic acid (EGTA), sodium tetraphenylborate (Wako, Osaka); adenosine 5'-triphosphate, disodium salt (Kojin, Tokyo); aconitine, choline kinase (Sigma, St. Louis, MO, USA); tetrodotoxin, crystalline 3X (Sankyo, Tokyo); and A23187 (Carbiochem, San Diego, CA, USA) were used.

RESULTS

Increasing and decreasing effects of aconitine on stimulation-evoked ACh release

Figure 1a shows the tritium overflow after the washout period in control experiments. Electrical stimulation-evoked tritium release from the mouse phrenic nerve-hemidiaphragm muscle preparations preloaded with ^{3}H-choline was induced by two consecutive electrical stimulation periods (S_1 and S_2). The electrically evoked increase in tritium release over the level of spontaneous output can be attributed to ^{3}H-ACh release from the nerve terminal, while tritium overflow at the resting state consists of 80% ^{3}H-choline and 20% ^{3}H-ACh (9, 12).

A low concentration of aconitine (0.1 μM) that was administered 10 min before S_2-stimulation period increased the evoked ^{3}H-ACh release, whereas at 1 μM, it decreased the release (Fig. 1, b and c). The concentration-response

Fig. 2. Concentration-response curves of aconitine for evoked ^{3}H-ACh release (S_2/S_1: closed symbols) and peak amplitude of tetanic tension (T_2/T_1: open symbols) in the mouse phrenic nerve-diaphragm muscle preparation. Aconitine was administered in various concentrations 10 min before S_2 as indicated in Fig. 1, b and c. Each point represents the mean value for relative ratios of ACh release (S_2/S_1) and tension (T_2/T_1) (n=4-5), and vertical lines represent ±S.E. T_1 and T_2 were averaged values of the 6th, 12th and 18th peak amplitudes of tetanus produced by S_1 and S_2 stimulation periods, respectively. Significant differences from the control (without drug) were analyzed by one way analysis of variance followed by Scheffe's test. **P<0.01.

curve of aconitine for ACh release (S_2/S_1) and peak amplitude of tetanic tension (T_2/T_1) are shown in Fig. 2. A biphasic curve for S_2/S_1 was found. Aconitine at 0.1 μM increased the S_2/S_1 ratio; and at concentrations above 0.3 μM, it decreased the ratio. The decrease in evoked ACh
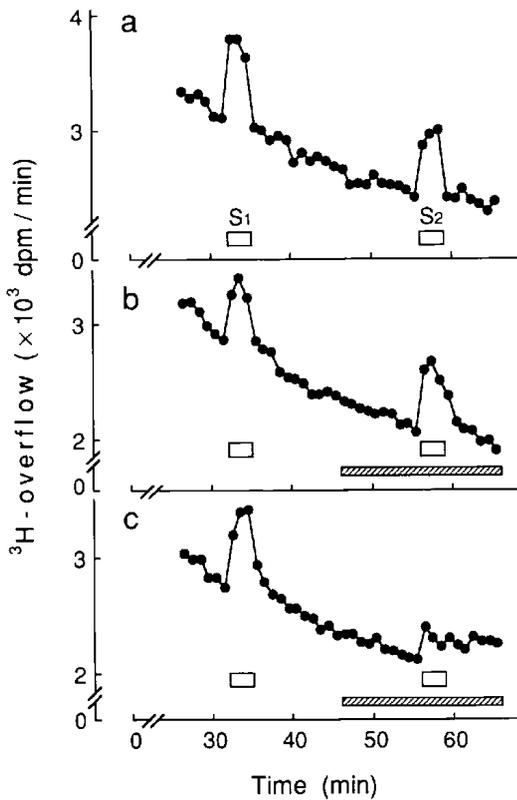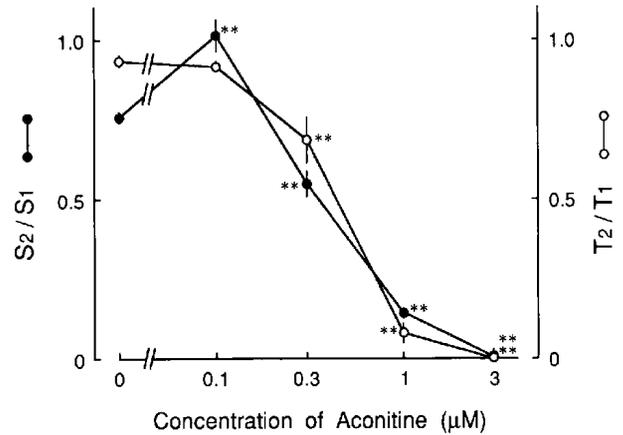
Fig. 1. The effect of aconitine on electrically evoked ^{3}H-acetylcholine (ACh) release from the mouse nerve-hemidiaphragm muscle preparation. The tissue was incubated for 1 hr with ^{3}H-choline (electrical stimulation for 40 min and rest 20 min) and then washed out for 1 hr. ^{3}H-ACh release was elicited by two stimulation periods (S_1, S_2: 50 Hz, trains of 40 stimuli applied every 10 sec for 3 min) as indicated by the open columns. The shaded column indicates the presence of aconitine (b: 0.1 μM, c: 1 μM). Each point represents the mean value (n=5). The horizontal axis indicates the time after the end of the washout period.

Table 1. Inhibitory effect of terodotoxin (TTX) on the aconitine-induced increase and decrease of stimulation-evoked acetylcholine release from the mouse phrenic nerve-diaphragm muscle preparation

Drugs	Concentration (nM)	S_2/S_1
1. nil	—	0.76±0.02
2. Aconitine	100	1.01±0.06**
3. TTX	5	0.58±0.04*
4. Aconitine +TTX	100 5	0.68±0.04
5. Aconitine	1000	0.14±0.03**
6. TTX	13	0.45±0.02**
7. Aconitine +TTX	1000 13	0.48±0.03

Aconitine and TTX were administered to the perfusate 10 and 15 min before S_2, respectively. Each value represents mean±S.E. of 5 experiments. The significance of differences was analyzed by two way analysis of variance followed by a studentized range test. *P<0.05, **P<0.01, vs nil. ††P<0.01, vs the corresponding concentration-group of aconitine in the absence of TTX. N.S.: not significant.

release was accompanied by simultaneous depression of the peak amplitude of tetanic tension. Aconitine at 0.3 and 1 μM decreased the muscle tension time-dependently (data not shown). Aconitine at 3 μM completely inhibited the tension. The IC_{50} (95% confidence limit) for S_2/S_1 and T_2/T_1 were 0.49 μM (0.43−0.57) and 0.46 μM (0.39 −0.55), respectively.
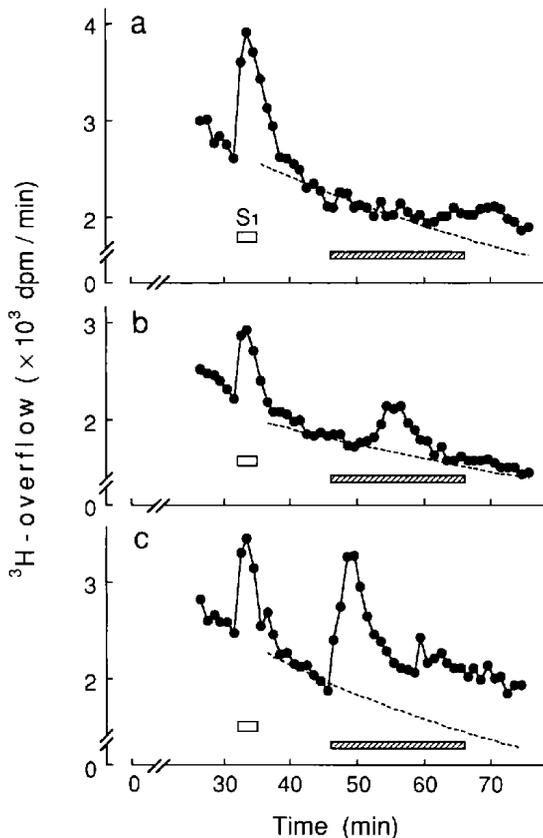
Inhibitory effect of TTX on aconitine-induced increase and decrease of evoked ACh release

TTX, a sodium channel blocker, inhibited the effect of aconitine on evoked ACh release (Table 1). Treatment with 5 nM TTX, which reduced ACh release per se, abolished the aconitine (0.1 μM)-induced increase in ACh release. The inhibitory effect induced by 1 μM aconitine was suppressed by TTX at 13 nM significantly, but not at 10 or 15 nM. TTX at 10 nM was less effective; and at 15
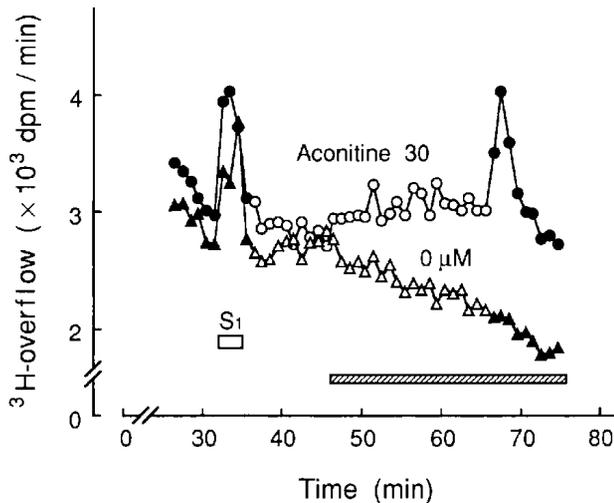

Fig. 3. The effect of aconitine on the spontaneous release of tritium radioactivity from the mouse phrenic nerve-hemidiaphragm preparation. The nerve was stimulated (50 Hz, trains of 40 stimuli with an interval of 10 sec) for 3 min as indicated by the open column. The shaded column indicates the presence of aconitine (a: 1 μM, b: 3 μM, c: 30 μM). Each point represents the mean value of tritium radioactivity (n = 3−4). The dotted line indicates the mean of expected outflows calculated from the control outflows without the drug. The horizontal axis indicates the time after the end of the washout period.

nM, its inhibitory effect was too marked to restore the S_2/S_1 value (data not shown). The values of the aconitine (0.1 or 1 μM) with TTX-treated group and TTX alone-treated group were not significantly different.

Increasing effect of aconitine on Ca^{2+}-dependent and -independent spontaneous ^3H-overflow

Aconitine increased the resting tritium overflow (Fig. 3). The tritium overflow enhanced by aconitine (10 μM) was separated into ^3H-choline and ^3H-ACh by the modified choline kinase method. ^3H-ACh release was mainly enhanced by aconitine (79±4% of the total ^3H-release, n=3). The increase in spontaneous release started about 15 min after administration of 1 μM aconitine (Fig. 3a), which followed after the inhibition of electrically evoked ACh. At a higher concentration (3 μM), the increase in the resting overflow occurred within 10 min and declined despite the presence of the drug (Fig. 3b). Electrical stimulation (S_2) that was applied 10 min after addition of 3 μM aconitine neither elicited further ACh release above the basal release nor muscle tension at all, suggesting that a complete block of the transmission occurred after the onset of the phasic spontaneous increase. The resting release was markedly enhanced at high concentrations of aconitine. Aconitine at 30 μM induced a continuous release at a low level after the sharp increase (Fig. 3c) and produced a phasic contraction (1.0−2.0 mN) (data not shown). The curves of tritium overflow in the presence of aconitine (46th−66th min) were quantified with two parameters: the peak and the total radioactivity of tritium overflow (Table 2). Aconitine increased the above both parameters in a concentration-dependent manner. At 1 μM, the peak amplitude of the release was not detected because the release continued increasing after the 20 min period. In the presence of TTX (1 μM), the amount of the

Table 2. Effect of aconitine on spontaneous release of tritium from the mouse phrenic nerve-diaphragm muscle preparation

Concentration (μM)	Peak (dpm)	Total (dpm)	n
0	—	0± 130	5
1	—	1097± 266	3
3	549± 88	3394± 611*	4
10	989±146	6682±1296**	4
30	1556± 92	14267± 591**	3

Aconitine was administered from the 46th−66th min after the end of the washout period. Peak amplitude and total amount of tritium release elicited by the drug for 20 min above the expected basal level were estimated. Each value was corrected using that of the control (without aconitine). Values are means±S.E. n: number of experiments. The significance of differences from the control was analyzed by one way analysis of variance followed by Scheffe's test. *P<0.05, **P<0.01.

Fig. 4. Ca^{2-}-deprivation effect on aconitine-induced increase in spontaneous release of tritium radioactivity from the mouse phrenic nerve-hemidiaphragm preparation. The stimulation (50 Hz, trains of 40 stimuli with an interval of 10 sec) was applied to the phrenic nerve as indicated by the open column. Ca^{2+} was omitted (closed symbols: 2.5 mM Ca^{2+}, open symbols: 0 mM Ca^{2+} containing 1 mM EGTA) 10 min before the addition of aconitine (circles: 30 μM, n=4). The shaded column indicates the presence of aconitine. Triangles indicate the control data without the drug (n=2). Each point represents the mean value. The horizontal axis indicates the time after the end of the washout period.

total radioactivity released by 10 μM aconitine decreased to the control level (-144 dpm, n=2).

The Ca^{2+}-dependence of the spontaneous release induced by aconitine (30 μM) was examined (Fig. 4). When the extracellular Ca^{2+} was removed 10 min before addition of aconitine by perfusion with 0 mM Ca^{2+} solution containing 1 mM EGTA, the sharp increase in tritium overflow disappeared, whereas the prolonged release was not affected. These data indicate that aconitine increased both Ca^{2+}-dependent and independent spontaneous release. The Ca^{2+}-dependent release is transient and Ca^{2+}-independent one is long lasting. The decline of the Ca^{2+}-dependent release is not due to inactivation of Na^+ influx to the nerve, because readmission of Ca^{2+} to the perfusate caused a sharp increase in the release that was suppressed by TTX (10 μM). A23187, a Ca^{2+} ionophore, at 20 μM increased the tritium overflow even after the depression of the Ca^{2+}-dependent spontaneous release (data not shown).

DISCUSSION

Neurotoxins that act on sodium channels are classified into five groups according to their receptor sites associated with the channels (13). Aconitine, like batracotoxin, veratridine and grayanotoxin binds to a second receptor

site (2). This toxin causes a persistent activation of sodium channels and depolarizes the cell membrane (3, 4). The details of the toxin-induced activation or inactivation of transmitter release are still unknown.

In this study, we demonstrated that aconitine at low concentration (0.1 μM) enhanced and at higher concentrations (0.3–3 μM) inhibited the electrical stimulation-evoked ^3H-ACh release. Muroi et al. (5) has shown that aconitine decreased the quantal content by inhibiting the nerve action potential without depolarizing the muscle membrane in the mouse phrenic nerve-diaphragm muscle preparation. They concluded that the reduction of ACh release results in the neuromuscular blockade. We found that IC_{50} values of aconitine for electrically evoked ACh release (S_2/S_1) and the peak amplitude of the tetanic tension (T_2/T_1) were substantially the same. This may indicate that the suppression of the muscle tension is attributable to the decrease in the release. Since the sodium channel blocker TTX, which binds to neurotoxin receptor site 1 on the channel, suppressed the facilitatory and inhibitory effect of aconitine, both effects seem to involve excessive activation of the channels in the nerve membrane.

High concentrations (3–30 μM) of aconitine increased spontaneous tritium release within 20 min in a concentration-dependent manner. Most of the tritium radioactivity released by aconitine originated from ^3H-ACh, likewise those by electrical nerve stimulation (9, 10) or by a high concentration of K^+ (12). TTX abolished the effect of aconitine, suggesting that a depolarization of the nerve cell membrane by excessive sodium channel activation increases the release. We found the aconitine-induced spontaneous release consisted of two different components: a phasic release that is inactivated within a few minutes and a low level of long lasting release. ACh is released from two different compartments in the cholinergic nerve terminal, as a quantal release from the vesicular store and a non-quantal release from the cytoplasmic one (14). The nerve action potentials trigger the quantal ACh release by a Ca^{2+}-dependent exocytosis (15). Under the resting condition, ACh is released mainly in the non-quantal, Ca^{2+}-independent manner, whereas the quantal release also occurs spontaneously, generating miniature end-plate potentials (16). The Ca^{2+}-dependent release seems to reflect the exocytotic release of vesicular ACh, and the Ca^{2+}-independent one may indicate a non-quantal molecular leakage of ACh (12). Omission of Ca^{2+} from the perfusate abolished only the aconitine-induced phasic release without affecting the long lasting one. This result indicates that both the components are attributed to different release mechanisms and compartments: probably the phasic release is from the vesicular store, and the long lasting one is from cytoplasmic compartment. Aconitine at

20–60 μM causes an increase in the frequency of miniature end-plate potential (6), which may correspond to the Ca^{2+}-dependent phasic release. Our result indicated that not only the Ca^{2-}-dependent, quantal release but the Ca^{2+}-independent, non-quantal release was increased by aconitine. Similar results have been demonstrated in the studies on high K^+ concentration-induced ACh release from *Torpedo* synaptosomes (17) and rat phrenic nerve (12). Therefore, membrane depolarization may elicit spontaneous ACh release from both the vesicular and cytoplasmic stores in the nerve terminal. Yu and Van der Kloot (7), however, found a significant decrease in non-quantal release in the presence of 10 μM aconitine by the electrophysiological method. The high concentration of aconitine has been shown to cause a gradual decline of the resting membrane potential (3, 5), which may result in apparent inhibition of non-quantal release. Subsequent Ca^{2+} addition 20 min after administration of aconitine (30 μM) increased the release and then the release decayed rapidly. This increasing effect was inhibited by TTX, indicating that sodium channels in the nerve in part remain activatable even when the quantal release is completely inactivated under the normal condition. Therefore, the decay of quantal ACh release is not attributable to the inactivation of the sodium channel itself. Since the Ca^{2+} ionophore A23187 still released a large amount of ACh after the cessation of quantal release, the inactivation may be neither due to the depletion of releasable ACh nor the inactivation of the release mechanism, but due to a limitation in Ca^{2+} entry. This explanation does not contradict the previous study (17) indicating that there is a depression of ACh release caused by Ca^{2+}-dependent inactivation of Ca^{2+} entry under prolonged K^+ depolarization. We showed that aconitine-induced inhibition of electrically evoked ACh release preceded the enhancement of spontaneous release. The prolonged activation of sodium channels by aconitine may abolish the conduction of nerve action potential, and causes the inhibition of the evoked release. Further activation of the channels may lead to membrane depolarization, which induces the spontaneous release, and then complete inactivation of quantal release occurs Ca^{2+}-dependently.

In conclusion, aconitine at a low concentration increases and at high concentrations decreases the electrical stimulation-evoked ACh release from the phrenic nerve-diaphragm muscle preparations of mice through the persistent activation of the voltage-sensitive sodium channels in motor nerve cell membrane. Further activation of the channels produced by high concentrations of aconitine enhances both Ca^{2+}-dependent (quantal) and -independent (non-quantal) spontaneous ACh release, and then complete inactivation of quantal release may occur Ca^{2+}-dependently.

REFERENCES

1   Catterall WA: Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annu Rev Pharmacol Toxicol **20**, 15–43 (1980)

2   Catterall WA: Cooperative activation of action potential Na^+ ionophore by neurotoxins. Proc Natl Acad Sci USA **72**, 1782–1786 (1975)

3   Schmidt H and Schmitt O: Effect of aconitine on the sodium permeability of the node of Ranvier. Pflugers Arch **349**, 133–148 (1974)

4   Grishchenko II, Naumov AP and Zubov AN: Gating and selectivity of aconitine-modified sodium channels in neuroblastoma cells. Neuroscience **9**, 549–554 (1983)

5   Muroi M, Kimura I and Kimura M: Blocking effects of hypaconitine and aconitine on nerve action potentials in phrenic nerve-diaphragm muscles of mice. Neuropharmacology **29**, 567–572 (1990)

6   Bozdagi O, Onur R and Ayata C: Effects of aconitine on transmitter release in the rat neuromuscular junction. Abstract of the XIIth International Congress of Pharmacology, p 20.1.11., Montreal (1994)

7   Yu SP and Van der Kloot W: Non-quantal acetylcholine release at mouse neuromuscular junction: effects of elevated quantal release and aconitine. Neurosci Lett **117**, 111–116 (1990)

8   Kimura I, Okazaki M, Uwano T, Kobayashi S and Kimura M: Succinylcholine-induced acceleration and suppression of electrically evoked acetylcholine release from mouse phrenic nerve-hemidiaphragm muscle preparation. Jpn J Pharmacol **57**, 397–403 (1991)

9   Kimura I, Okazaki M and Kimura M: Streptozocin-diabetes modifies acetylcholine release from mouse phrenic nerve terminal and presynaptic sensitivity to succinylcholine. Jpn J Pharmacol **62**, 35–41 (1993)

10   Visi ES, Somogyi GT, Nagashima H, Duncalf D, Chaudhry IA, Kobayashi O, Goldiner PL and Foldes FF: Tubocurarine and pancuronium inhibit evoked release of acetylcholine from the mouse hemidiaphragm preparation. Br J Anaesth **59**, 226–231 (1987)

11   McGee R Jr, Smith C, Christian C, Mata M, Nelson P and Nirenberg M: A new capillary tube system for measuring the uptake and release of materials from cultured cells. Anal Biochem **101**, 320–326 (1980)

12   Wessler I and Steinlein O: Differential release of [^{3}H]-acetylcholine from the rat phrenic nerve-hemidiaphragm preparation by electrical nerve stimulation and by high potassium. Neuroscience **22**, 289–299 (1987)

13   Catterall WA: Cellular and molecular biology of voltage-gated sodium channels. Physiol Rev **72**, S15–S48 (1992)

14   Thesleff S: Different kinds of acetylcholine release from the motor nerve. Int Rev Neurobiol **28**, 59–88 (1986)

15   Katz B and Miledi R: The effect of calcium on acetylcholine release from motor nerve terminals. Proc R Soc Lond B, **161**, 496–503 (1965)

16   Visi ES and Vyskočil F: Changes in total and quantal release of acetylcholine in the mouse diaphragm during activation and inhibition of membrane ATPase. J Physiol (Lond) **286**, 1–14 (1979)

17   Birman S and Meunier FM: Inactivation of acetylcholine release from *Torpedo* synaptosomes in response to prolonged depolarizations. J Physiol (Lond) **368**, 293–307 (1985)