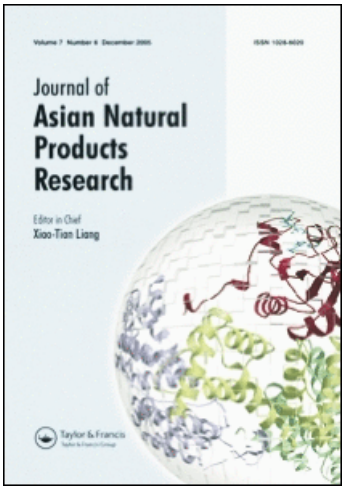

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COUNTER EFFECTS OF HIGENAMINE AND CORYNEINE, COMPONENTS OF ACONITE ROOT, ON ACETYLCHOLINE RELEASE FROM MOTOR NERVE TERMINAL IN MICE

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The counter effects of higenamine and coryneine, components of aconite root, on acetylcholine (ACh) release from motor nerve terminals in the mouse phrenic nerve–diaphragm muscle preparation were studied by a radioisotope method. Both nerve-evoked release and spontaneous release of [^{3}H]-ACh from the preparation preloaded with [^{3}H]-choline were measured. The change in the tetanic tension of muscle was simultaneously recorded in the same preparation. Higenamine (10 μM) augmented both the nerve-evoked and spontaneous ACh releases, and the muscle tension. The effects were inhibited by pretreatment with propranolol (10 μM), a β-adrenoceptor antagonist. Coryneine reduced the nerve-evoked release of ACh, accelerated the decay of tetanic tension (tetanic fade) at 30 μM, and it depressed the peak amplitude of tetanic tension at a higher concentration of 100 μM. These results suggest that of the two components contained in aconite root, higenamine increases ACh release via activation of β-adrenoceptor, and conversely coryneine depresses ACh release by preferentially acting at motor nerve terminal.

Keywords: Acetylcholine release; Higenamine; Coryneine; Aconite; Phrenic nerve–diaphragm muscle preparation

INTRODUCTION

Aconite is frequently prescribed in Kampo-Hozai (traditional Sino-Japanese medicines) in combined forms such as Keishi-ka-jutsubu-to which is used to relieve muscle pain [1]. Higenamine and coryneine are two of the

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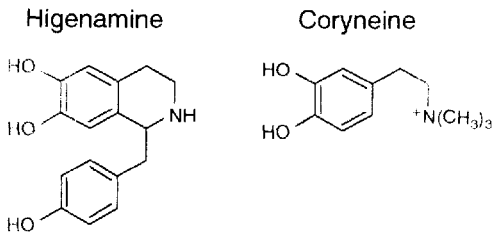

FIGURE 1 The chemical structures of higenamine and coryneine.

components derived from aconite root [2,3]. Higenamine, a tetrahydroisoquinoline derivative (Fig. 1), has a neuromuscular blocking action [4] in addition to a cardiotonic action by activation of β-adrenoceptors [5] and subsequent increase in Ca^{2+} influx [6]. The affinity of higenamine for the β-adrenoceptors in turkey erythrocyte membrane is similar to that of isoproterenol on cyclic adenosine monophosphate generation [7]. On the other hand, coryneine, a quaternary ammonium derivative of dopamine (Fig. 1), has also a neuromuscular blocking action by inactivation of nicotinic acetylcholine (ACh) receptors following the activation at endplates [8]. Until now, the presynaptic action of these components in neuromuscular synapse is unclear. Hence, we investigated the effects of higenamine and coryneine on ACh release from motor nerve terminals in the mouse phrenic nerve–diaphragm muscle preparation, using a radioisotope method.

RESULTS AND DISCUSSION

The tritium overflow and the nerve-evoked tritium release were induced by two consecutive electrical stimulation periods (S_1 and S_2) after the washout period in the mouse phrenic nerve–hemidiaphragm muscle preparations which were preloaded with [^{3}H]-choline (Fig. 2A). The electrical nerve stimulation-evoked increase in tritium release over the level of spontaneous output is attributed to [^{3}H]-ACh from the nerve terminal [9], while tritium overflow at the resting state consists of 20% [^{3}H]-ACh and 80% [^{3}H]-choline [10]. In our previous paper [11], we compared electrically evoked ACh release from mouse phrenic nerve diaphragm muscle preparation determined by [^{3}H]-efflux counting, with that determined by radioimmunoassay. We have found that S_2/S_1 value determined by [^{3}H]-efflux is about 0.7, though S_2/S_1 value determined by radioimmunoassay is almost 1.0. This finding means that the amount of [^{3}H]-efflux released into

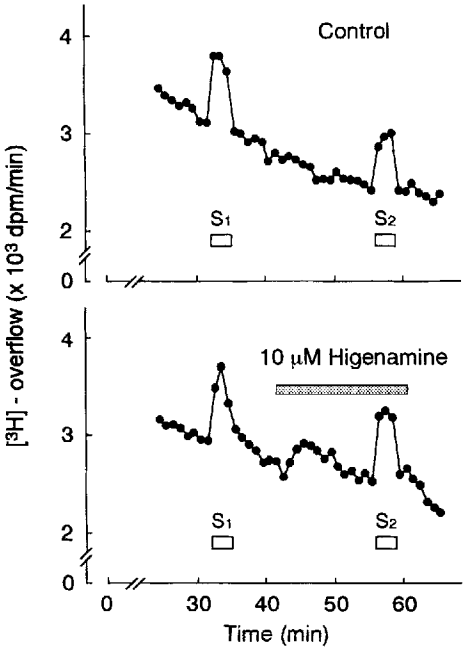

FIGURE 2A The augmented effect of higenamine on electrically-evoked [^{3}H]-acetylcholine (ACh) release from the mouse phrenic nerve–hemidiaphragm muscle preparation. Tritium radioactivity overflowed from the preparation was measured. [^{3}H]-ACh release was elicited by two stimulation periods (S_1 and S_2) as indicated by the open columns. The shaded column indicates the presence of 10 μM higenamine. Each point represents the mean value ($n = 6$ in control and $n = 3$ in higenamine). The horizontal axis indicates the time after the end of the washout period.

perfusates decreases during S_2, but the amount of ACh released does not decrease during it. Higenamine (10 μM) that was administered 15 min before S_2-stimulation period increased both the evoked and spontaneous [^{3}H]-ACh releases (Fig. 2A). The concentration–response curves of higenamine for the evoked ACh release (S_2/S_1) and peak amplitude of tetanic tension (T_2/T_1) are shown in Fig. 2B. Steep and gentle bell-shaped curves in the range of 1–100 μM were obtained for S_2/S_1 and T_2/T_1, respectively. These enhancing effects of higenamine were completely blocked by pretreatment with propranolol (10 μM), a β-adrenoceptor antagonist (Fig. 2B). Propranolol had no alone effect on the values of S_2/S_1 and T_2/T_1 at a concentration used in this study. Higenamine elicited the potentiation of tetanic tension at more than 3 μM, and increased the nerve-evoked ACh release at 10 μM. Therefore, the augmenting effect of higenamine on tetanic tension may not result from increase in ACh release from motor nerve terminal, but from

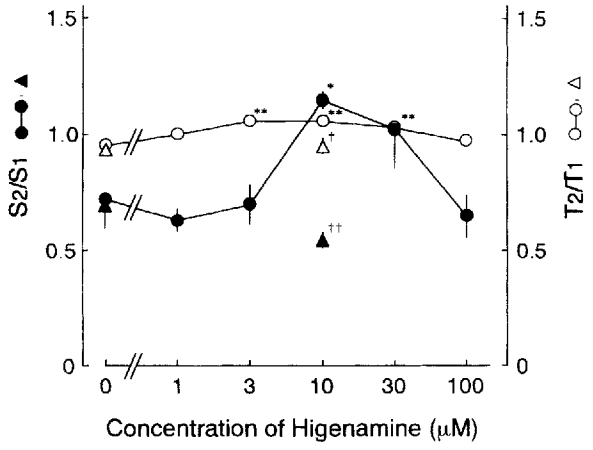

FIGURE 2B Concentration-response curves of higenamine for evoked [^{3}H]-ACh release (S_2/S_1: closed symbols) and peak amplitude of tetanic tension (T_2/T_1: open symbols) in mouse phrenic nerve diaphragm muscle preparation. Each point represents the mean value for relative ratios of ACh release and tetanic tension in experimental number of 3–6, and vertical lines represent mean ± SEM. The triangles indicate the experiments in the presence of 10 μM propranolol, which was applied 20 min before S_2. Significant differences from the control (without drug) were analyzed by one way ANOVA followed by Scheffe's test, $^{*}P < 0.05$ and $^{**}P < 0.01$. Significant differences between the values with and without propranolol were analyzed by unpaired t-test, $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$.

direct stimulating action to skeletal muscle membrane, via activation of β-adrenoceptors. The effects of higenamine on ACh release and tetanic tension were found to diminish rather at higher concentrations (30–100 μM). This phenomenon may be due to desensitization (down regulation) of β-adrenoceptors as described on the biphasic effect of isoproterenol on ACh release from rat phrenic nerve [12]. We showed that higenamine increased not only nerve-evoked ACh release but also spontaneous ACh release. The effect on spontaneous ACh release was also completely inhibited by pretreatment with propranolol (data not shown), which may be similar to the increasing effects of adrenaline and noradrenaline on frequency of miniature endplate potential [13].

Coryneine (100 μM) that was administered 10 min before S_2-stimulation period depressed the evoked [^{3}H]-ACh release, whereas it did not affect the spontaneous tritium overflow (Fig. 3A). And then, acceleration of tetanic fade was obviously observed (Fig. 3B). Aconitine, which is a main component of aconite root [2,3] and a moderator of voltage-dependent sodium channel [14], depressed preferentially the peak amplitude of tetanic tension without an acceleration of tetanic fade. Succinylcholine, which blocks with

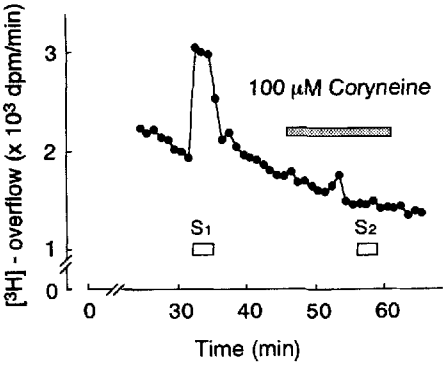

FIGURE 3A The depressed effect of coryneine on electrically-evoked [^{3}H]-acetylcholine (ACh) release from the mouse phrenic nerve–hemidiaphragm muscle preparation. Tritium radioactivity overflowed from the preparation was measured. [^{3}H]-ACh release was elicited by two stimulation periods (S_1 and S_2) as indicated by the open columns. The shaded column indicates the presence of 100 μM coryneine. Each point represents the mean value ($n = 3$). The horizontal axis indicates the time after the end of the washout period.

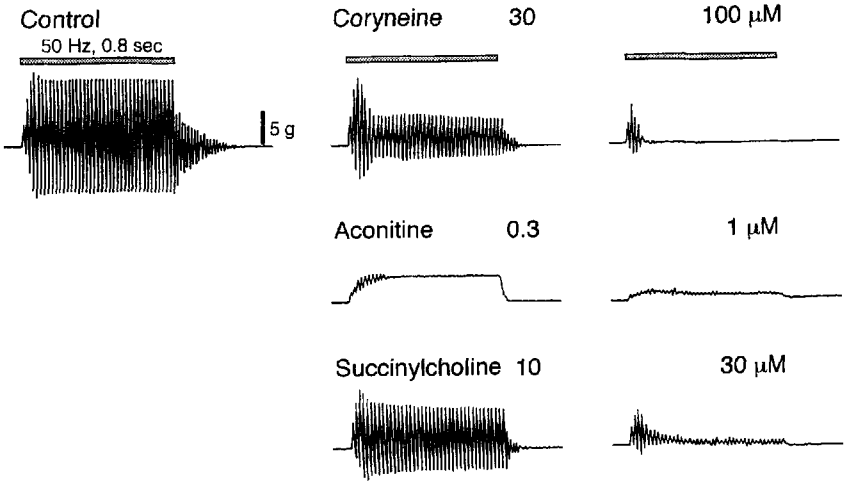

FIGURE 3B Typical data showing the accelerative effect of coryneine on tetanic fade in mouse phrenic nerve–hemidiaphragm muscle preparation, compared with those of aconitine and succinylcholine. Tetanic contraction of the muscle was elicited by electrical stimulation of the nerve (50 Hz for 0.8 s) which are indicated by bars.

the same potency both pre- and postsynaptic nicotinic ACh receptors [14], induced to the same extent both the depression of peak amplitude of tetanic tension and the acceleration of tetanic fade. The concentration–response curves of coryneine for the evoked ACh release (S_2/S_1), peak amplitude of

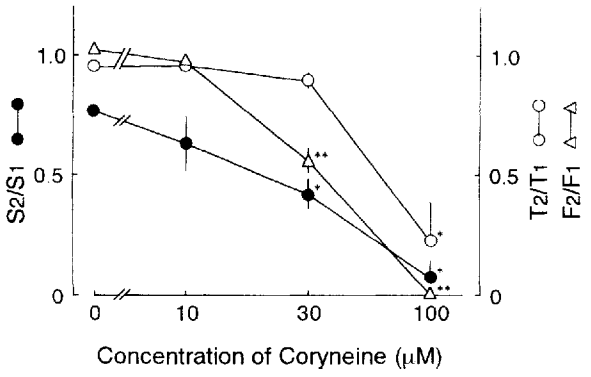

FIGURE 3C Concentration–response curves of coryneine for evoked [^{3}H]-ACh release (S_2/S_1: closed circles), peak amplitude of tetanic tension (T_2/T_1: open circles) and tetanic fade (F_2/F_1: open triangles) in mouse phrenic nerve–diaphragm muscle preparation. Each point represents the mean value for relative ratios of ACh release, tetanic tension and tetanic fade in experimental number of 3–6, and vertical lines represent mean + SEM. Significant differences from the control (without drug) were analyzed by one way ANOVA followed by Scheffe's test. $^*P < 0.05$ and $^{**}P < 0.01$.

tetanic tension (T_2/T_1) and extent of decay of tetanic tension (tetanic fade, F_2/F_1) are shown in Fig. 3C. Coryneine (30–100 μM) depressed significantly the evoked ACh release in a concentration-dependent manner, which is parallel to the accelerating effect on the tetanic fade. It depressed significantly the peak amplitude of tetanic tension only at a higher concentration of 100 μM. These results suggest that coryneine depresses the neuromuscular transmission mainly by inhibiting the nerve-evoked ACh release, but not by reducing the excitation of postsynaptic membrane. We have previously reported that coryneine elicits depolarization at endplate of mouse skeletal muscle, which is blocked by pancuronium, a nicotinic ACh receptor antagonist [8]. Therefore, coryneine may interact with nicotinic ACh receptors of not only muscle type existing on skeletal muscle but also neuronal type existing on motor nerve terminal. Tetanic fade reflects mainly the inhibition of an accelerative feedback mechanism via activation of presynaptic neuronal type nicotinic ACh receptors [15]. Hence, coryneine may have higher affinity to a neuronal type of nicotinic ACh receptor compared with that to a muscle type one.

In conclusion, these results suggest that of the two components contained in aconite root, higenamine increases ACh release via activation of β-adrenoceptor, and conversely coryneine depresses ACh release preferentially acting at motor nerve terminal.

EXPERIMENTAL SECTION

Male ddY mice (7- 9-week-old, 30–41 g) were decapitated under light ether anesthesia 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 (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) gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C.

Measurement of Radioactivity for [^{3}H]-ACh in the Perfusate

A radioisotope method was used to measure the release of ACh without cholinesterase inhibitors. The detail was described in our previous paper [16]. The above preparation was incubated for 60 min in 2 ml Krebs solution containing methyl-[^{3}H]-choline (370 kBq). To facilitate the uptake of [^{3}H]-choline into motor nerve terminals, the phrenic nerve was electrically stimulated (50 Hz for 0.8 s administered every 10 s, 0.2 ms duration, 1–10 V) for 40 min. For the next 20 min, the preparation was allowed to rest. The preparation was then washed with Krebs solution for 60 min to remove the excess [^{3}H]-choline. The perfusion rate was 1 ml/min. The periods of the above electrical stimulation for 3 min were started at 8 min (S_0) and 32 min (S_1) after the washout period. Additionally, S_2, which started 21 min after the end of S_1, was applied to examine the effect of higenamine or coryneine on the electrically evoked ACh release. Higenamine and coryneine were administered to the perfusing solution 6 and 11 min after S_1 (15 and 10 min before S_2), respectively. Samples were collected every minute from 9 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 [^{3}H] 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.

Measurement of Peak Amplitude and Decay of Tetanic Tension

The isometric contraction of the diaphragm muscle was simultaneously measured with a force displacement transducer (SB1T; Nihon Kohden, Tokyo, Japan) and recorded (Linea Recorder, WR3701; Graphtec, Tokyo, Japan). The resting tension was adjusted to 500 mg. The effects of drugs on the peak amplitude and decay of the tetanic tension were represented as T_2/T_1 and F_2/F_1, respectively, where T and F were averaged values of the peak amplitudes and the ratio of minimum to maximum amplitude, respectively, in 6th, 12th and 18th tetanus for a 3-min stimulation period. T_1 and F_1 were produced by S_1 stimulation periods, and T_2 and F_2 were produced by S_2 stimulation periods.

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 unpaired t-test. $P < 0.05$ was adopted as the level of significance.

Drugs

Methyl-[^{3}H]-choline chloride (555 GBq/mmol; Amersham, Buckinghamshire, England) was used. (±)-Higenamine hydrochloride and coryneine chloride ([2-(3,4-dihydroxyphenyl)ethyl]trimethylammonium chloride) were synthesized and gifted by Prof. T. Momose and the late Prof. T. Koizumi (Department of Synthetic Organic Chemistry in Toyama Medical and Pharmaceutical University), respectively. Aconitine (Sigma, St. Louis, MO, USA) and succinylcholine dichloride (Nacalai Tesque, Kyoto, Japan) were used.

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