Appearance of Tolerance in the Increase of Contractile Muscle Tension by Ambenonium in Rats

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The relationship between the concentration of ambenonium (AMB), a selective and reversible acetylcholinesterase (AChE) inhibitor, in plasma and the potentiation of contractile muscle tension was investigated using a sciatric nerve-muscle preparation of rat. The developed isometric contraction was enhanced dose-dependently after i.v. administration of low doses (5—20 nmol/kg) of AMB, but the contraction was weakened when AMB was administered at high doses (100—1000 nmol/kg), and the concentration-effect relationship was bell-shaped. The muscle contraction profile after 50 nmol/kg administration without previous administration, with 20 nmol/kg and with 50 nmol/kg administered previously were quite different from each other. These findings suggest that the potentiation of contractile muscle tension by AMB may be acutely tolerated and the concentration-effect relationship may change time-dependently.

Keywords ambenonium; cholinesterase inhibitor; muscle tension; tolerance

Reversible cholinesterase (ChE) inhibitors are used as the first choice of therapy to treat myasthenia gravis. ChE inhibitors elevate the acetylcholine (ACH) concentration at the synaptic cleft of the neuromuscular junction by acetylcholinesterase (AChE) inhibition, and intensify contractile muscle tension. However, the relationship between the concentration of ChE inhibitors in plasma and the enhancement of contractile muscle tension has not been clearly elucidated. Chan and Calvey1) reported a positive correlation between the concentration of pyridostigmine in plasma and neuromuscular transmission function in five myasthenic patients. In contrast, Davison et al.2) investigated the same concentration-effect relationship in nine myasthenic patients, and found only two patients who demonstrated a significant positive correlation between the pyridostigmine concentration in plasma and the clinical evaluation of muscle tension; a negative correlation was found in one patient. Aquilonius et al.3) reported on the relationship between the concentration of ChE inhibitors and the muscle response after i.v. administration of neostigmine and pyridostigmine. In their study, positive correlations were found between the drug concentration in plasma and responses to lower concentrations, but the effect declined with increases in plasma concentration at the higher concentration, again suggesting a bell-shaped concentration-effect relationship. However, the severity of disease states and dose of drugs administered were not controlled in these clinical studies with myasthenic patients. Therefore, basic animal studies under controlled conditions are required.

In this study, we investigated the time-dependent relationship between the concentration of ambenonium (AMB), a potent AChE inhibitor,4) in plasma and the enhancement of contractile muscle tension after i.v. administration to rats.

MATERIALS AND METHODS

Chemicals and Reagents AMB chloride was generously supplied by Nippon Shoji Co. (Osaka, Japan). Benzo-
the blood was taken at 7.5, 15, 22.5 and 30 min after the first administration. The plasma sample was obtained immediately by centrifugation and was stored at −20 °C until analysis.

**Pharmacokinetic Analysis** To estimate the pharmacokinetic parameters, A, B, α and β, the concentration of AMB in plasma obtained in all single bolus dose experiments were simultaneously fitted to Eq. 1 by the non-linear least squares method. The total body clearance (\( \text{CL}_{\text{tot}} \)) and the steady state volume of distribution (\( \text{V}_{\text{ss}} \)) and the elimination half life (\( t_{1/2} \)) were calculated according to Eqs. 2, 3 and 4, respectively.

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C_p = \text{Dose} \left( A \cdot \exp(-\alpha \cdot t) + B \cdot \exp(-\beta \cdot t) \right)
\]

\[
\text{CL}_{\text{tot}} = \frac{\alpha \cdot \beta}{A \cdot \beta + B \cdot \alpha}
\]

\[
V_{\text{ss}} = \frac{A \cdot \beta^2 + B \cdot \alpha^2}{(A \cdot \beta + B \cdot \alpha)^2}
\]

\[
t_{1/2} = \frac{\ln 2}{\beta}
\]

where \( C_p \) is the concentration of AMB in plasma, Dose is the i.v. dose administered and \( t \) is the time after administration.

**Assay of AMB in Plasma** The concentration of AMB in plasma after administering 300—1000 nmol/kg was determined by the HPLC method. Briefly, 1 nmol of benzilinium bromide as the internal standard, 1 ml of 1 M HCl and 4 ml of CH₂Cl₂ were added to the 0.2 ml of plasma. The mixture was shaken for 10 min and centrifuged. One ml of the upper phase was transferred to another tube, and 11 ml of CH₂Cl₂ and 0.2 ml of 1 M HClO₄ were added. The mixture was shaken for 10 min, then separated by centrifugation. After the upper aqueous phase had been removed, 10 ml of organic phase were transferred to another tube and 0.2 ml of 1 M NaClO₄—1 M NaOH (4:3) solution was added. The mixture was shaken for 10 min and separated by centrifugation. Then 9 ml of the lower organic phase was evaporated to dryness. The dried residue was dissolved in a mobile phase and injected onto a column.

The HPLC was carried out with an LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an SPD-6A V spectrophotometer (Shimadzu, Kyoto, Japan) set at 217 nm. The column was a stainless-steel tube packed with Senshu gel 7C₁₈H (Senshu Kagaku, Tokyo, Japan). The column temperature was maintained at 40 °C. The mobile phase was 35% acetonitrile in water containing 20 mm sodium octanesulphonate, 2.5 mm tetramethylammonium chloride and 10 mm KH₂PO₄, and the pH was adjusted to 3.0 with concentrated sulfuric acid. The mobile phase was degassed before use and pumped at a flow rate of 2.0 ml/min. The detection limit of the HPLC method was as low as 100 nm.

Samples obtained from the lower dose studies (5—100 nmol/kg) were assayed by the enzymatic method. Endogenous plasma ChE was removed by filtration with an MPS-3 centrifuge micro partition system (Amicon, U.S.A.) at 4 °C, 2000 g for 15 min. From 0.5 to 1 ml of plasma, 0.1 ml of plasma filtrate was obtained. The plasma filtrate was transferred to a plastic tube, then 25 μl of 1 mg/ml 5,5’-dithiobis-2-nitrobenzoic acid in a phosphate buffer (50 mm, pH 7.2), and 0.1 ml of 200 unit/l AChE were added. After preincubation at 37 °C for 5 min, 25 μl of 25 mg/ml acetylthiocholine was added. Exactly 5 min after the addition of the substrate, the enzyme reaction was stopped by the addition of 0.6 ml of iced 0.1 μM neostigmine. The sample was placed on ice for 5—10 min, and then absorbance at 412 nm was measured in a spectrophotometer. A blank sample was prepared by substituting the phosphate buffer and drug free plasma for the enzyme solution and sample plasma, respectively. The enzyme reaction velocity was calculated by subtracting the absorbance of the blank from that of the sample. For calibration curves, AMB was added to the drug free plasma at final concentrations of 1, 2, 5, 10, 20, 50, 100 and 200 nm. The calibration curves were prepared by plotting the reciprocal of the reaction velocity versus concentration of AMB in the range of 1—20 and 20—200 nm, respectively.

**RESULTS**

The plasma concentration profiles of AMB and the increasing ratios of contractile muscle tension after i.v. bolus administration are shown in Figs. 1 and 2, respectively. The concentration—time curves could be fitted to the two exponential equation (see Method), and no dose dependency was observed (Fig. 1). The total body clearance, the steady state volume of distribution and the elimination half-life were 6.01 ± 1.81 ml/min/kg, 0.494 ± 0.176 l/kg, and 63.5 ± 43.2 min, (optimal estimates ± S.E., \( n = 24 \)) respectively.

The increase in tension in the contractile muscle following the administration of low doses (5—20 nmol/kg) was slow, and the increasing ratio was positive and dose dependent (Fig. 2A). In contrast, a transient increase followed by a rapid decrease of the contractile muscle tension was observed after the administration of high doses (100—1000 nmol/kg), and the contractile tension at 60 min

![Fig. 1. The Plasma Concentration Profile of AMB after i.v. Bolus Administration](image-url)
Fig. 2. The Increase of Muscle Tension after i.v. Bolus Administration of AMB

was decreased as the dose increased (Fig. 2B). Figure 3 shows the relationship between the plasma concentration and the potentiation of muscle tension after the i.v. bolus administration of AMB. A bell-shaped relationship was observed, suggesting the depressive effect of AMB at high concentrations.

To confirm the decrease in contractile potency of AMB at high concentrations, additional doses of 50 nmol/kg of AMB were administered intravenously 15 min after the first administration of AMB. The concentration time profiles of AMB in plasma after the additional ad-

Fig. 3. The Relationship between the AMB Concentration and the Potentiation of Muscle Tension after i.v. Bolus Administration of AMB

Fig. 4. The Plasma Concentration of AMB after Additional Administration

Fig. 5. The Increase of Muscle Tension after Additional i.v. Administration of AMB

ministration were very close to the simulation lines of the pharmacokinetic parameters estimated by a single bolus administration study (Fig. 4). On the other hand, the pattern of the contractile muscle tension was considerably different from the single dose study. Following a preceding administration of 20 nmol/kg, a slight increase in muscle tension was observed after the second administration, though the extent of tension increase was much smaller than that after single doses of 50—100 nmol/kg. Furthermore, a decrease in contractile muscle tension was observed after the second administration following the preceding administration of 50 nmol/kg of AMB (Fig. 5).

DISCUSSION

Reversible ChE inhibitors, such as AMB, are the first choice for the therapeutic treatment of myasthenia gravis. However, the relationship between the clinical effects of these drugs and their concentration in plasma has not been clearly elucidated. A positive correlation was found be-
between the effect and the concentration in plasma in some patients, but not in other patients. To establish a rational drug therapy, it is necessary to understand the concentration–effect relationship based on the controlled study of the pharmacokinetics and pharmacodynamics of these inhibitors in experimental animals, without variations due to the difference in disease states, drug dosage, and/or other factors which complicate human studies.

As shown in Fig. 3, a bell-shaped relationship between plasma concentration and the increasing ratio of contractile muscle tension after the i.v. bolus administration of AMB was observed. Aquilonius et al. also reported a similar relationship in myasthenic patients after the i.v. bolus administration of pyridostigmine or neostigmine. In their study, a positive relationship between plasma concentration and effect was seen when the plasma concentration was lower than 30–60 ng/ml for pyridostigmine and 5–15 ng/ml for neostigmine, but higher concentrations gave a negative correlation between plasma concentration and effect. Similarity of the bell-shaped concentration–effect relationship among these drugs suggested that it is a general characteristic of acetylcholinesterase inhibitors.

Thus, the plasma concentration after an additional dose of 50 nmol/kg AMB is substantially the same as the estimated value from the single dose study (Fig. 3). Therefore, the pharmacokinetic behavior of AMB is not thought to be changed by any preceding administration of AMB. By contrast, the profiles of contractile muscle tension were considerably different between the first and the second dose. Especially, the increasing ratio of muscle tension after the additional administration of AMB following the preceding administration of 50 nmol/kg was slightly decreased. (Fig. 5) One possibility for this difference may be a desensitization of the ACh receptor induced by the first administration of AMB. Acute desensitization of an ACh receptor at a high concentration of AMB may lead to the bell-shaped and time-dependent concentration–effect relationship. Another possibility could be due to between difference of the drug concentration in plasma and in the receptor site, as Aquilonius et al. discussed. If the transport rate of the drug to the effective site is slow, the concentration of the drug at the effective site would be high and show a direct antagonistic effect on the ACh receptor, similar in its bell-shape and time dependent relationship. A small volume of distribution (0.491/kg) was inconsistent with the accumulation of drug in the deep tissues, but the possibility of accumulation to very small effective site such as synaptic cleft was not excluded. Therefore, the mechanism of change in the concentration–effect relationship remains to be elucidated.

The reduction of the ratio of potentiation of contractile muscle tension after the first administration of AMB in this study may play a role in the concentration independence of the clinical effect of ChE inhibitors. Pyridostigmine potentiated a muscle twitch caused by the electronic stimulation of a nerve at 0.2–0.4 mm and depressed it at more than 0.8 mm. In that study, pyridostigmine did not affect membrane potential or muscle action potential, but a change in the number and/or properties of conducting channels at the synaptic cleft were indicated. Pyridostigmine was also found to inhibit the binding of ACh; therefore, the mechanism of the twitch depression caused by pyridostigmine was thought to involve a weak agonist action or the formation of desensitized receptor–complex intermediates.

In this study, the plasma concentration of AMB at 15 min after 20 nmol/kg administration was similar to those in myasthenic patients reported previously. However the plasma concentration of AMB is about 10 times higher than values reported by other investigators. For other ChE inhibitors, pyridostigmine and neostigmine, bell-shaped plasma concentration–effect relationships were observed during the clinical treatment of myasthenic patients. The pyridostigmine concentration which induced the desensitization of ACh receptors (50–100 μM) was considerably higher than the plasma concentration in myasthenic patients (30–700 nm). Furthermore, neuromuscular function did not change after repeated doses of pyridostigmine at a dose of 90 mg/d for 8 d. It may be necessary to consider the possibility that adverse effects relate to the wide variations in the effective concentrations of ChE inhibitors.

REFERENCES