Mechanism of the differential sensitivity in the rat adductor and abductor laryngeal muscles to a non-depolarizing neuromuscular blocker

M. IGARASHI AND H. IWASAKI

Summary
We have compared the neuromuscular blocking effects of tubocurarine at pre- and postsynaptic sites in the lateral cricoarytenoid muscle (LCA) (one of the adductor muscles of the vocal cords) and in the posterior cricoarytenoid muscle (PCA) (sole abductor muscle of the vocal cords) of the rat. Fine wire electrodes were inserted into both muscles and evoked compound electromyographic (EMG) responses measured by supramaximal stimulation of the recurrent laryngeal nerves. End-plate potentials (EPP), mean quantal content and carbachol sensitivity to tubocurarine in both muscles were measured using intracellular microelectrodes. Tubocurarine produced a concentration-dependent reduction in the EMG responses, EPP amplitude, mean quantal content and carbachol sensitivity. The LCA muscle was more resistant to tubocurarine than the PCA muscle in EPP amplitude, mean quantal content and carbachol sensitivity, suggesting unequal pre- and postsynaptic sensitivity for inhibition of elicited acetylcholine release, reduction in EPP amplitude and loss of evoked muscle action potentials. Examination of muscle fibre composition demonstrated that the LCA muscle contained a significantly higher fraction of slow twitch muscle fibres than PCA muscle. However, the sizes of the fibres were similar in both muscles. We conclude that the mechanism of unequal sensitivity to a non-depolarizing neuromuscular blocker in the LCA and PCA muscles may be explained by differential sensitivities at the pre- and postsynaptic sites of the neuromuscular junction. (Br. J. Anaesth. 1995; 75: 339–343)

Key words
Neuromuscular block, tubocurarine, Larynx, muscles. Rat.

Differential sensitivity to non-depolarizing neuromuscular blockers has been reported in humans [1–3] and dogs [4] between the laryngeal and peripheral skeletal muscles. The intrinsic laryngeal muscles comprise the lateral cricoarytenoid (LCA), posterior cricoarytenoid (PCA), interarytenoid, aryepiglottis, thyroarytenoid, thyroepiglottis, vocalis and cricothyroid muscles [5]. The LCA muscle adducts the cord by internally rotating the arytenoid cartilage, thus closing the glottis; it is the strongest adductor muscle of the larynx. The PCA muscle abducts the cord by external rotation of the arytenoid and opens the glottis; it is the sole abductor muscle of the larynx [5].

We have examined the responses of the LCA and PCA muscles to a non-depolarizing neuromuscular blocker using pre- and postsynaptic electrophysiological microelectrodes. As the intrinsic laryngeal muscles are composed of a heterogeneous mixture of slow and fast twitch muscles [6], we also studied muscle fibre composition of both muscles using histochemical staining.

Materials and methods
The study was approved by the Sapporo Medical University Animal Ethics Committee. All experiments were carried out using dissected recurrent laryngeal nerve-intrinsic laryngeal muscle preparations from 31 Wistar rats, 16–20 weeks old, weighing 330–356 g (mean 340 g).

Muscle preparations
The rats were killed with pentobarbitone 50 mg kg\(^{-1}\) i.p. The neck was opened at the midline and the larynx exposed, avoiding injury to the muscles, nerves and vessels. The larynx with the attached recurrent laryngeal nerves was dissected free, placed in a chamber and perfused with oxygenated Krebs solution. The preparations were pinned together onto the Sylgard (Dow Corning, USA) base of a 5-ml tissue bath which was perfused continuously (10–15 ml min\(^{-1}\) at 24–25 °C) with modified Krebs solution of the following composition (mmol litre\(^{-1}\)): NaCl 135; KCl 5; CaCl\(_2\) 2; NaHCO\(_3\) 15; Na\(_2\)HPO\(_4\) 1; MgCl\(_2\) 1; glucose 11. The solution was maintained at pH 7.3 and bubbled with 95 % oxygen and 5 % carbon dioxide [7, 8]. Contraction of the LCA and PCA muscles by supramaximal stimulation of the recurrent laryngeal nerves was confirmed before the experiment was started.

M. IGARASHI, MD, H. IWASAKI, MD, PhD, Department of Anesthesiology, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo 060, Japan. Accepted for publication: February 28, 1995.

Correspondence to M. I.
MEASUREMENT OF EMG

In order to obtain evoked compound electromyographic (EMG) responses of the intrinsic laryngeal muscles, thin intramuscular stainless steel electrodes (0.3 mm in diameter, MT Giken Co., Japan) stripped of insulation for 0.3 mm along the tips were inserted into the bellies of the LCA and PCA muscles. The recurrent laryngeal nerve was stimulated by a single supramaximal train of rectangular pulses (4.0–10.0 mA, duration 0.2 ms). The evoked compound EMG responses of the LCA and PCA muscles were recorded onto paper with a Neuromatic 2000 (Dantec, DIASA, Denmark). After obtaining stable evoked EMG responses from both muscles for at least 30 min, the tissues were exposed to tubocurarine (Wako Chemical Co., Japan) 1.0 × 10⁻⁸ to 1.0 × 10⁻⁴ mol litre⁻¹ for 30 min. The effects of tubocurarine were completely reversible. The decrease in EMG amplitude produced by tubocurarine was expressed as a percentage of control values, and changes in the LCA and PCA muscles were compared. We also recorded the time to peak evoked compound action potential in both muscles.

ELECTROPHYSIOLOGICAL STUDIES

The neuromuscular junction in the intrinsic laryngeal muscles after administration of tubocurarine was assessed using intracellular microelectrodes. Preparations were bathed in glycerol–Krebs solution 400 mmol litre⁻¹ [9] for 90 min and then the solution in the bath chamber was changed to the normal Krebs solution. Single barrel pipettes (4–8 MΩ resistance) with tip diameters less than 1.0 μm were filled with KCl 3.0 mol litre⁻¹ and used as micro-electrodes. An Olympus (Olympus Optics Co., Japan) binocular compound microscope fitted with a 20 × long-range working distance objective with a total magnification of 400 × was used to view the preparation and enabled the electrodes to be positioned on the muscles with micromanipulators. We used results from muscles with a resting potential of less than −65 mV.

MEASUREMENT OF END-PLATE POTENTIALS (EPP)

Pairs of platinum wires were embedded and filled with Krebs solution and recurrent laryngeal nerves were sucked into the electrodes [10]. The nerves were stimulated to elicit EPP using a SEN-3201 (Dantec, DISA, Denmark). After obtaining stable EPP responses from both muscles for at least 30 min, the tissues were exposed to tubocurarine (Wako Chemical Co., Japan) 1.0 × 10⁻⁸ to 1.0 × 10⁻⁴ mol litre⁻¹ for 30 min. The effects of tubocurarine were completely reversible. The decrease in EMG amplitude produced by tubocurarine was expressed as a percentage of control values, and changes in the LCA and PCA muscles were compared. We also recorded the time to peak evoked compound action potential in both muscles.

MEASUREMENT OF CARBACHOL SENSITIVITY

Carbachol sensitivity is a more useful variable than acetylcholine sensitivity for analysis of the postsynaptic effect of non-depolarizing neuromuscular blockers at the neuromuscular junction, because carbachol is not broken down by choline esterase [13]. Carbachol (carbamylcholine chloride, Katayama Chemical Co., Japan) potential was elicited by iontophoretic application using single-barrel capillaries with a diameter of 1.0 mm containing a glass fibre. Carbachol 3.0 mol litre⁻¹ (backing current 1.0–2000 nA, 10–100 ms, every 0.5–2.0 s) was applied to the end-plate via iontophoretic electrodes of resistance 30–60 MΩ. A backing current of more than 30 nA was required to prevent any increase in holding current caused by carbachol leakage [7, 14]. Pulses of 500-ms duration and between 100 and 400 nA in amplitude were used to apply carbachol to the end-plate from the electrode tip. The iontophoretic electrode was manipulated carefully around the end-plate until it was placed in the optimum region, where currents of less than 20 ms duration could be elicited. The currents had rise times of less than 3.0 ms and decayed exponentially between 85 and 15% of their peak amplitude with time constants of 3.0–6.0 ms.

The capillaries were filled with KCl 3.0 mol litre⁻¹ and inserted into the muscle fibres at the end-plate to record membrane potentials induced by applied carbachol. We used data from muscles in which resting potentials ranged from −65 to −80 mV, with rise times of 15 ms [15]. The ratio of carbachol potentials (mV) to delivered charge (nC) during iontophoresis (mV nC⁻¹) [16] was calculated in control and tubocurarine-treated preparation. Data for tubocurarine are presented relative to control.

ACTOMYOSIN ATPASE STAINING OF RAT LARYNGEAL MUSCLES

LCA and PCA muscle pieces were mounted on brass chucks, oriented for cross-sectioning and frozen in liquid isopentane. Serial sections were cut on a
In order to improve fibre separation, some sections were incubated sequentially in alkaline buffer (pH 11.0, 11.1, 11.2) containing CaCl₂ 0.18 mol litre⁻¹ and acid buffer (pH 3.8, 3.9, 4.0, 4.1). Areas with good staining and correct transverse fibre sections were selected for counting with a Nikon standard microscope (Nikon Co., Japan).

### Statistical Analysis

Electrophysiological and histochemical data are expressed as mean (SEM). Data were compared by two-way, repeated measures ANOVA combined in part with the unpaired t test. P < 0.05 was considered statistically significant.

### Results

#### Effect of Tubocurarine on EMG Responses

EMG responses in LCA and PCA muscles were depressed by tubocurarine in a dose-dependent and reversible (eight of ten) manner (figs 1, 2). Data analysis was therefore based on eight LCA and PCA preparations. Time to peak evoked compound action potentials was 1.10 (0.09) ms for LCA and 0.98 (0.08) ms for PCA muscle (P > 0.05).

#### Effects of Tubocurarine on EPP

EPP in LCA and PCA muscles were depressed in a concentration-dependent manner by tubocurarine, with PCA muscle being more sensitive (fig. 3). Statistically significant differences in the degree of depression of EPP amplitude between the LCA and PCA muscles were observed at >7.5 × 10⁻⁷ mol litre⁻¹ (fig. 4).

The effects of tubocurarine on quantal release in LCA and PCA muscles were determined by 1.0-Hz stimulation of recurrent laryngeal nerves. Although the mean quantal contents in both the LCA and PCA muscles were depressed in a tubocurarine concentration-dependent manner, LCA muscle showed higher mean quantal content than PCA muscle at most concentrations of tubocurarine tested (table 1).

#### Carbachol Sensitivity

Carbachol sensitivities in the two muscles were also depressed in a dose-dependent manner by tubocurarine. At concentrations in excess of 5 × 10⁻⁷ mol litre⁻¹, there was a significant difference between LCA and PCA muscles, with PCA muscle being more sensitive to tubocurarine (fig. 5).

### Discussion

Several factors may underlie the differential effects of neuromuscular blockers on the intrinsic laryngeal muscles. These include perfusion [18, 19], temperature, muscle fibre composition [20], size of muscle fibre [21], and numbers and densities of acetylcholine receptors [22]. In addition to simple block of postsynaptic acetylcholine recognition sites at the neuromuscular junction, non-depolarizing neuromuscular blockers are well known for their presynaptic action [23, 24]. It has been reported that the PCA muscle is better perfused [25] than the adductor pollicis muscles, and that the difference in perfusion among the intrinsic laryngeal muscles contributes to the differences in sensitivity to blockers [2]. However, this cannot account for the different responses to tubocurarine observed in the present study.

In general, cut-fibre preparations or high concentrations of magnesium have been used in EPP

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**Figure 1** Effect of tubocurarine (TC mol litre⁻¹) on the amplitude of the evoked compound electromyograph in the lateral cricoarytenoid (LCA) and posterior cricoarytenoid (PCA) muscles.

**Figure 2** Depression of EMG responses in the lateral cricoarytenoid (LCA) and posterior cricoarytenoid (PCA) muscles after administration of tubocurarine (TC). Data (mean, SEM) are expressed as percentage of control (n = 8). *P < 0.05, **P < 0.01 compared with PCA muscle.
studies to prevent muscle movement after the motor nerve is stimulated [26]. However, as the intrinsic laryngeal muscles of the rat are too small for this technique, we used glycerol-treated preparations to elicit EPP in this study. It has been reported that excitation of these muscles is uncoupled from contraction, while nerve ending transmitter release, and pre- and postsynaptic sensitivities are maintained [9]. At tubocurarine concentrations less than $5 \times 10^{-7}$ mol litre$^{-1}$, EPP were not observed in the LCA muscle (fig. 3) because action potentials were still present. In contrast, EPP in the PCA muscle were observed at tubocurarine concentrations of $5 \times 10^{-7}$ mol litre$^{-1}$ (fig. 3). These data indicate that the differential sensitivity to tubocurarine between the two muscles observed in both the EMG and EPP studies is associated with unequal sensitivity of acetylcholine receptors to tubocurarine at the neuromuscular junction.

Differential sensitivity to tubocurarine may occur at pre- or postsynaptic sites. These were investigated further. The presynaptic effect is commonly estimated by mean quantal content analysis, usually calculated by the direct method: EPP = MEPP [8]. However, the variance method [27] was used to evaluate the presynaptic effects of tubocurarine on LCA and PCA muscles in our study because MEPP are not observed at higher concentrations of tubocurarine. It is accepted that mean quantal content, $m$, determined by variance, is in agreement with that determined directly [27]. Quantal release has been measured at 200–400 quanta per pulse in curarized preparations of the rat diaphragm [7, 11, 27]; thus the presently observed mean quantal content of 289 (46) at tubocurarine $5 \times 10^{-7}$ mol litre$^{-1}$ in the PCA muscle is in agreement. A significant difference was observed in mean quantal content between the two muscles after administration of the same concentrations of non-depolarizing blocker (table 1). The
LCA muscle showed significantly higher mean quantal content than the PCA muscle. This presynaptic effect could be the result of inhibition of transmitter mobilization by tubocurarine, leading to diminished quantal release. Bowan [23] reported that the acetylcholine receptors on the nerve terminal are important in controlling transmitter release under physiological conditions, and that the negative feedback effect of acetylcholine limits the amount of transmitter release and may therefore help to conserve intracellular acetylcholine [8].

Carbachol sensitivities in LCA and PCA muscles decreased proportionally with increase in tubocurarine concentration (fig. 5), although carbachol sensitivity in the LCA muscle was more resistant to tubocurarine. The unequal sensitivity to tubocurarine in LCA and PCA muscles may be explained not only by postsynaptic but also by presynaptic differences in the sensitivity of acetylcholine receptors to non-depolarizing blockers at the neuromuscular junction.

It has been reported that muscles with high contents of slow twitch muscle fibres (type I muscle fibre) are more resistant to neuromuscular blockers than those of predominantly fast (type II) twitch fibres [22]. Our histochemical study demonstrated a significant difference in fibre composition between the two muscles, with LCA muscle possessing more slow twitch fibres than PCA muscle (table 2). This may account for some of the unequal sensitivity to blockers. Recently, an inverse association has been described between the neuromuscular effect of blockers and muscle fibre size [21]. However, in our study there was no significant difference in fibre size of the laryngeal muscles.

It was noted that the difference in the postsynaptic membrane between slow and fast twitch muscles was caused by the prominent raised area of slow muscle fibres (about three times larger than in the fast muscles); nevertheless, release sites were about equal in number in both muscles [28]. This difference in the postsynaptic membrane might also be a factor in the different sensitivity to neuromuscular blockers. However, further studies are needed to explain the observed discrepancy in sensitivity to tubocurarine.

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References