

## Interaction of Edrophonium with Muscarinic Acetylcholine $M_2$ and $M_3$ Receptors

Yasuto Tanito, M.D.,\* Takaaki Miwa, M.D., Ph.D.,† Masayuki Endou, M.D., Ph.D.,‡ Yoshihumi Hirose, M.D., Ph.D.,§ Masahiro Gamoh, M.D.,|| Haruaki Nakaya, M.D., Ph.D.,# Fukuichiro Okumura, M.D., Ph.D.\*\*

**Background:** It has been reported that edrophonium can antagonize the negative chronotropic effect of carbachol. This study was undertaken to evaluate in detail the interaction of edrophonium with muscarinic  $M_2$  and  $M_3$  receptors.

**Methods:** A functional study was conducted to evaluate the effects of edrophonium on the concentration–response curves for the negative chronotropic effect and the bronchoconstricting effect of carbachol in spontaneously beating right atria and tracheas of guinea pigs. An electrophysiologic study was conducted to compare the effects of edrophonium on carbachol-, guanosine triphosphate (GTP) $\gamma$  S-, and adenosine-induced outward  $K^+$  currents in guinea pig atrial cells by whole cell voltage clamp technique. A radioligand binding study was conducted to examine the effects of edrophonium on specific [ $^3$ H]N-methylscopolamine (NMS) binding to guinea pig atrial ( $M_2$ ) and submandibular gland ( $M_3$ ) membrane preparations, and on atropine-induced dissociation of [ $^3$ H]NMS.

**Results:** Edrophonium shifted rightward the concentration–response curves for the negative chronotropic and bronchoconstricting effects of carbachol in a competitive manner. The  $pA_2$  values for cardiac and tracheal muscarinic receptors were 4.61 and 4.03, respectively. Edrophonium abolished the carbachol-induced outward current without affecting the GTP $\gamma$  S- and adenosine-induced currents in the atrial cells. Edrophonium inhibited [ $^3$ H]NMS binding to  $M_2$  and  $M_3$  receptors in a concentration-dependent manner. The pseudo-Hill coefficient values and apparent dissociation constants of edrophonium for  $M_2$  and  $M_3$  receptors were 1.02 and 1.07 and 21 and 34  $\mu$ M, respectively. Edrophonium also changed dissociation constant values of [ $^3$ H]NMS without affecting its maximum binding capacities.

**Conclusion:** Edrophonium binds to muscarinic  $M_2$  and  $M_3$  receptors nonselectively, and acts as a competitive antagonist.

PARASYMPATHOMIMETIC effects of acetylcholinesterase inhibitors such as neostigmine and edrophonium are thought to be theoretically inevitable, and side effects, including bradycardia, bronchoconstriction, and increased bowel movement, are encountered when used to reverse the muscle weakness induced by nondepolarizing muscle relaxants.<sup>1-3</sup> In the clinical setting, however, the bradycardic effect of edrophonium was reported to be less potent than that of neostigmine,

although the doses of the drugs to reverse the effects of the muscle relaxants to a comparable degree were used.<sup>4</sup> Consistent with the clinical data, Backman *et al.*<sup>5</sup> suggested that the bradycardic effect of edrophonium is solely a result of the anticholinesterase effect, whereas neostigmine possesses an additional effect on cholinergic receptors within the cardiac parasympathetic pathway, thereby producing profound bradycardia. Recently, we also demonstrated that neostigmine at clinically relevant concentrations decreased the spontaneously beating rate of isolated guinea pig right atria in an atropine-sensitive manner, whereas the therapeutic concentrations of edrophonium inhibited the negative chronotropic effect of carbachol without any direct chronotropic effects.<sup>6</sup>

Molecular cloning studies have identified five subtypes of muscarinic acetylcholine receptors (mAChRs) ( $m_1$ – $m_5$ ) expressed in various tissues, while pharmacologic studies revealed four different subtypes of mAChRs ( $M_1$ – $M_4$ ) based on their binding affinities to specific ligands.<sup>7</sup> Although recent studies have shown the existence of  $M_1$  receptors in ventricular cells,  $M_2$  is thought to be the predominant subtype of cardiac mAChR and mediates the typical cardiodepressant effects of parasympathetic activation such as bradycardia.<sup>8-10</sup> Coexpression of  $M_2$  and  $M_3$  receptors seems to be a common feature of smooth muscle cells in various tissues such as airway and intestinal smooth muscles.<sup>11</sup> Although the amount of  $M_3$  receptors is less than that of  $M_2$  receptors in these tissues,<sup>7</sup> the  $M_3$  receptors are usually responsible for the direct contractile effect of muscarinic agonists on smooth muscles, including guinea pig and human airway smooth muscles.<sup>12-15</sup>

Our previous findings that edrophonium inhibited the negative chronotropic effect of carbachol suggested the antagonism of edrophonium to  $M_2$  mAChRs.<sup>6</sup> Therefore, in the present study we investigated the interaction of edrophonium with the peripheral muscarinic receptor subtypes  $M_2$  and  $M_3$  in detail by means of functional, electrophysiologic, and radioligand binding experiments.

### Materials and Methods

All experiments were conducted in conformity with the “Guiding Principles for Research Involving Animals and Human Beings” of Yokohama City University School of Medicine.

\* Staff Anesthesiologist, Yokosuka Kyosai Hospital, Yokosuka, Japan. † Staff Anesthesiologist, Kanagawa Pediatric Hospital, Yokohama, Japan. ‡ Lecturer, \*\* Professor and Chairman, Department of Anesthesiology, Yokohama City University School of Medicine. § Chief Anesthesiologist, || Staff Anesthesiologist, Kanagawa Cardiovascular and Respiratory Center, Yokohama, Japan. # Professor and Chairman of Pharmacology, Chiba University School of Medicine, Chiba, Japan.

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Address reprint requests to Dr. Endou: Department of Anesthesiology, Yokohama City University School of Medicine, Yokohama 236-0004, Japan. Address electronic mail to: endo@ac7.mopera.ne.jp. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

### Functional Studies

#### Effect of Edrophonium on the Negative Chronotropic Response to Carbachol in Guinea Pig Atria.

Male guinea pigs weighing 250–350 g were killed by cervical dislocation during light anesthesia with diethyl ether. After midline thoracotomy, the heart was rapidly excised and placed in a dissection dish filled with oxygenated Krebs-Henseleit solution of the following composition: 119.0 mM NaCl, 2.5 mM CaCl<sub>2</sub>, 4.8 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 24.9 mM NaHCO<sub>3</sub>, and 10.0 mM glucose. The spontaneously beating right atrium was carefully dissected and mounted vertically in a 12-ml double-walled glass chamber filled with Krebs-Henseleit solution, gassed continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.4), and maintained at 36°C. The lower end of the right atrium was fixed on a hook, and the upper end was connected to an isometric force-displacement transducer (model UL-10GR; Minebea, Nagano, Japan) by a silk thread. Changes in tension were recorded with a pen recorder (model Recti-Horiz 8K; Sanei-Sokki, Tokyo, Japan) through a preamplifier (model AS2103, Sanei-Sokki). Diastolic tension was adjusted to 0.5 g. The spontaneous beating rate was counted on a chart recording of its developed tension. The preparation was equilibrated for at least 60 min before the commencement of the experiments.

The concentration–response curve for the negative chronotropic effect of carbachol was determined in a cumulative manner by increasing its concentration in steps of 0.5 M log units. At the completion of the concentration–response curve, the preparation was washed thoroughly. After beating rate returned to the predrug level, one concentration of edrophonium was applied. The drug at concentrations used in the present study (100 μM, 300 μM, and 1 mM) did not change the basal beating rate significantly. After the incubation time of 30 min, the concentration–response curve for carbachol was obtained in the presence of edrophonium. We confirmed that repetitive application of carbachol to the preparation did not affect the concentration–response curve for its negative chronotropic effect. The concentration of carbachol causing half-maximal response in the absence and presence of various concentrations of edrophonium was estimated from log-probit plots of the individual response *versus* concentrations. Affinity measurement for edrophonium was estimated by Schild plot analysis.<sup>16</sup>

**Effects of Edrophonium on the Contractile Response of Carbachol in Guinea Pig Trachea and Human Bronchus.** Male guinea pigs were killed as described above. The trachea was removed and placed in a dissection dish filled with oxygenated Krebs-Henseleit solution. The trachea was opened longitudinally opposite the tracheal smooth muscle and cut into transverse strips that included three cartilage rings. The epithelium was removed by rubbing the surface gently

with a swab. Two preparations were obtained from each trachea, and one of them served as a time-matched control to check the change in the sensitivity of the preparation during the repeated application of carbachol. The tracheal strip was mounted transversely in a 12-ml water-jacketed glass chamber, and change in the isometric tension was recorded as described above. Initial tension was adjusted to 0.5 g. After the preparation was equilibrated for 60 min, carbachol at a concentration of 1 μM was added repeatedly until the stable response of the preparation to carbachol was obtained.

The concentration–response curves for the contractile effect of carbachol were determined in the absence and presence of edrophonium at concentrations of 100 μM, 300 μM, and 1 mM. Edrophonium at a concentration of 100 μM increased the basal tension of the trachea slightly but consistently, although this effect became obscure once the preparation had been exposed to a high concentration of carbachol. The increase in the tension was abolished by atropine (1 μM), suggesting that the mechanism might be related to the inhibition of hydrolysis of acetylcholine liberated spontaneously from the cholinergic nerve terminals. The drug at higher concentrations did not further increase the basal contractile force. The concentration–response curves obtained during the second and third application of carbachol slightly shifted rightward compared with the first response curve, although the maximal responses of the preparation to carbachol did not change. Therefore, the EC<sub>50</sub> value of carbachol in the presence of edrophonium was corrected based on the difference between the EC<sub>50</sub> values obtained during the first and second or third application of carbachol in the time-matched control preparation. Affinity measurement for edrophonium was estimated by Schild plot analysis.<sup>16</sup>

Human lung tissue was obtained at lobectomy performed for lung carcinoma after obtaining informed consent and institutional approval. A macroscopically normal part of the lobe resected was excised within 5 min of resection and immersed in cold Krebs-Henseleit solution previously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The tissue was transported to the laboratory within 20 min. Macroscopically normal bronchus (2–3-mm ID) was dissected free of blood vessels and parenchyma. The bronchus was cut into rings (3 mm in width) and mounted for isometric tension recording in a 12-ml water-jacketed chamber. Initial tension was adjusted to 0.5 g, and the change in tension was recorded as described above. The concentration–response curve for the contractile effect of carbachol was determined in a cumulative manner in the absence and presence of 1 mM edrophonium. The change in sensitivity of the bronchus to the second application of carbachol was confirmed using three other preparations. The ratio of EC<sub>50</sub> value of the second application to that of the first application was 1.53 ± 0.14 (n = 3). The dose ratio was corrected based on this

value when the  $pK_B$  value for edrophonium was calculated.

#### *Electrophysiologic Studies*

Single atrial cells of guinea pig heart were isolated by an enzymatic dissociation method, as previously described.<sup>17</sup> Briefly, the heart was removed from an open-chest guinea pig anesthetized with pentobarbital sodium and mounted on a modified Langendorff perfusion system for retrograde perfusion of the coronary circulation with a normal HEPES-Tyrode solution. The perfusion medium was changed to a nominally  $Ca^{2+}$ -free Tyrode solution and then to the solution containing 0.02% wt/vol collagenase (Wako, Osaka, Japan). After digestion, the heart was perfused with a high  $K^+$ , low  $Cl^-$  solution (modified Kraftbrühe solution).<sup>18</sup> The atrial tissue was cut into pieces in the modified Kraftbrühe solution. The cell suspension in the Kraftbrühe solution was filtered through a 100- $\mu$ m pore, stainless steel mesh and stored in a refrigerator (4°C) for later use. The composition of the normal HEPES-Tyrode solution was as follows: 143 mM NaCl, 5.4 mM KCl, 1.8 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , 0.33 mM  $NaH_2PO_4$ , 5.5 mM glucose, and 5.0 mM HEPES-NaOH buffer (pH 7.4). The composition of the modified Kraftbrühe solution was as follows: 70 mM KOH, 50 mM l-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM  $KH_2PO_4$ , 3 mM  $MgCl_2$ , 10 mM glucose, 10 mM EGTA, and 10 mM HEPES-KOH buffer (pH 7.4).

Whole cell membrane currents were recorded by the patch clamp method.<sup>19</sup> Single atrial cells were placed in a recording chamber (1-ml volume) attached to an inverted microscope (model IMT-2; Olympus, Tokyo, Japan) and superfused with the HEPES-Tyrode solution at a rate of 10 ml/min. The temperature of the external solution was kept constant at  $36.0 \pm 1.0^\circ C$ . Patch pipettes were made from glass capillaries with a diameter of 1.5 mm by use of a vertical microelectrode puller (model PB-7; Narishige, Tokyo, Japan). They were filled with an internal solution, and the resistance was 2–3 M $\Omega$ . The composition of the pipette solution was as follows: 110 mM potassium aspartate, 20 mM KCl, 1.0 mM  $MgCl_2$ , 5.0 mM potassium adenosine triphosphate, 5.0 mM potassium phosphocreatine, 10 mM EGTA, and 5.0 mM HEPES-KOH buffer (pH 7.4). Guanosine triphosphate (GTP; 100  $\mu$ M) or GTP $\gamma$ S (100  $\mu$ M) was also added to the pipette solution. The free  $Ca^{2+}$  concentration in the pipette solution was adjusted to pCa 8 according to the calculation by Fabiato and Fabiato<sup>20</sup> with the correction of Tsien and Rink.<sup>21</sup> After gigaohm seal between the tip of the electrode and the cell membrane was established, the membrane patch was disrupted by more negative pressure to make whole cell voltage clamp mode. The electrode was connected to a patch clamp amplifier (model CEZ-2300; Nihon Koden, Tokyo, Japan). Recording signals were filtered at 2-kHz bandwidth, and series resistance was compensated. Command pulse signals were generated by 12-bit

digital-to-analog converter controlled by PCLAMP software (Axon Instrument, Inc., Foster City, CA). Current signals were digitized and stored on the hard disc of an IBM-compatible computer. A liquid junction potential between the internal solution and the bath solution of  $-8$  mV was corrected.

The mAChR-operated  $K^+$  current ( $I_{K,ACh}$ ) was activated by the extracellular application of 1  $\mu$ M carbachol or 10  $\mu$ M adenosine in the GTP-loaded atrial cells or by the intracellular loading of GTP $\gamma$ S, a nonhydrolyzable GTP analog, in atrial cells held at  $-50$  mV. The effects of 1 mM edrophonium on the  $I_{K,ACh}$  activated in the three different ways were examined. The activated  $I_{K,ACh}$  gradually decayed despite the continuous presence of the activators, probably because of desensitization. Assuming that the current decay was a linear function of time, the percent inhibition of  $I_{K,ACh}$  was calculated from the difference between the current level that was extrapolated from the current decay before the application of edrophonium and that changed by edrophonium.

#### *Radioligand Binding Experiments*

**Membrane Preparation.** Male guinea pigs weighing 250–500 g were killed by cervical dislocation during light anesthesia with diethyl ether. The hearts and salivary submandibular glands (SGs) were removed and rinsed in ice-cold Tris-HCl buffer. The atrial tissues were carefully dissected from the hearts. The tissues were minced with scissors and homogenized by polytron for 15 s in 5 vol ice-cold Tris-HCl buffer. The buffer composition (pH 7.4, 4°C) was 50 mM Tris-HCl 50 and 5 mM EDTA. The homogenates were centrifuged at 1,000 $g_{max}$  for 10 min at 4°C. The supernatants were filtered through a single layer of cheesecloth and centrifuged again. Membrane fractions in the supernatant were concentrated by centrifugation at 100,000 $g_{max}$  for 30 min at 4°C. The final pellets were resuspended in ice-cold Tris-HCl buffer and stored at  $-80^\circ C$  until used. The protein content was determined by the method of Lowry *et al.*<sup>22</sup> with bovine serum albumin as standard.

**Saturation Binding Assay.** The binding of the specific muscarinic antagonist [ $^3$ H]N-methyl-scopolamine ([ $^3$ H]NMS) (80 Ci/mmol, New England Nuclear, Boston, MA) was evaluated by a rapid filtration method<sup>23</sup> in the absence and presence of edrophonium at concentrations of 30 and 100  $\mu$ M. The membrane suspensions were diluted in an incubation medium (50 mM Tris-HCl, 0.5 mM EDTA, pH 7.4) to yield a final protein concentration of 0.2–0.4 mg/ml. [ $^3$ H]NMS was prepared in the incubation medium, and an aliquot of membrane suspension (100  $\mu$ l) was incubated with various concentrations of [ $^3$ H]NMS in a final volume of 200  $\mu$ l. The preparations were incubated for 70 min at 32°C. Incubation was terminated by adding 5 ml cold incubation medium (4°C) to the entire incubation mixture, followed by rapid filtration over Whatman GF/C glass filters (Maidstone,

United Kingdom). Each filter was washed three times with 5 ml cold incubation medium (4°C). The radioactivity trapped on the filter was counted in 5 ml Aquazol-2 (Packard, Meriden, CT) in a liquid scintillation counter. All values in binding experiments are average of triplicates. Nonspecific binding was defined as binding in the presence of 1  $\mu\text{M}$  atropine. The equilibrium dissociation constant ( $K_d$ ) and the maximum binding capacity ( $B_{\text{max}}$ ) were calculated from plots according to the method of Scatchard.<sup>24</sup>

**Competition Binding Assay.** The membrane suspension (100  $\mu\text{l}$ ) was incubated in triplicate with 200 pM [<sup>3</sup>H]NMS and various concentrations of edrophonium. The incubation was performed for 70 min at 32°C and terminated as described above. The data were analyzed by the iterative least-squares curve-fitting program described by Munson and Rodbard.<sup>25</sup> Results were analyzed to identify the presence of one or two classes of binding sites by comparing the sum of squares of residuals for each model. The most appropriate model was determined by statistical analysis using an F test of the sum of squares of the residuals. Apparent dissociation constant ( $K_i$ ) values were calculated by the method of Cheng and Prusoff.<sup>26</sup> In some experiments, the competition experiments were performed using 500 pM [<sup>3</sup>H]NMS.

**Dissociation Kinetic Studies.** Dissociation of specific [<sup>3</sup>H]NMS binding was measured at 32°C after incubation of cardiac membrane suspension (100  $\mu\text{l}$ ) with 200 pM [<sup>3</sup>H]NMS to equilibrium for 70 min in accordance with the method of Waelbroeck<sup>27</sup> with some modification. At time 0, a solution containing 1  $\mu\text{M}$  atropine with or without edrophonium at various concentrations was added to the incubation mixture. Residual binding of [<sup>3</sup>H]NMS was determined 20 min later. The degree of [<sup>3</sup>H]NMS dissociation from the receptors after the addition of atropine with edrophonium was indicated as a percentage of the specific [<sup>3</sup>H]NMS binding dissociated by 1  $\mu\text{M}$  atropine alone. The 20-min incubation with 1  $\mu\text{M}$  atropine dissociated almost completely 200 pM [<sup>3</sup>H]NMS from the binding site, as shown previously.<sup>23</sup>

## Drugs

The following drugs were used: edrophonium chloride, carbamylcholine chloride, atropine sulfate, adenosine (Sigma Chemical Co., St. Louis, MO). All chemicals were dissolved in distilled water.

## Statistics

Values are given as mean  $\pm$  SD. Comparison of variables obtained during a concentration-response curve was made by one-way analysis of variance followed by the Scheffé *t* test. Analysis by Student *t* test was performed for unpaired observation. The slope of Schild plot was analyzed with respect to difference from unity and 95% confidence limits of the slope according to Brown and Hollander.<sup>28</sup>  $P < 0.05$  was considered statistically significant.

## Results

### *Influence of Edrophonium on the Parasympathomimetic Effects of Carbachol*

The muscarinic receptor agonist carbachol decreased the spontaneous beating rate of the guinea pig right atria in a concentration-dependent manner. Edrophonium shifted the concentration-response curve for the negative chronotropic effect of carbachol to the right in a parallel manner (fig. 1). The  $\text{EC}_{50}$  values of carbachol in the absence of edrophonium and in its presence at concentrations of 100  $\mu\text{M}$ , 300  $\mu\text{M}$ , and 1 mM were  $0.22 \pm 0.06 \mu\text{M}$  ( $n = 17$ ),  $0.94 \pm 0.23 \mu\text{M}$  ( $n = 4$ ),  $2.48 \pm 0.55 \mu\text{M}$  ( $n = 5$ ), and  $9.26 \pm 4.18 \mu\text{M}$  ( $n = 8$ ), respectively. The slope of the regression line ( $0.96 \pm 0.05$ ) obtained from the Schild plot was not significantly different from unity. The  $\text{pA}_2$  value of edrophonium was 4.61.

Carbachol contracted the guinea pig tracheal smooth muscle in a concentration-dependent manner (fig. 2A). Edrophonium shifted the concentration-response curve for the contractile effect of carbachol to the right in a parallel manner without affecting the maximum force of contraction produced by carbachol. The  $\text{EC}_{50}$  values of

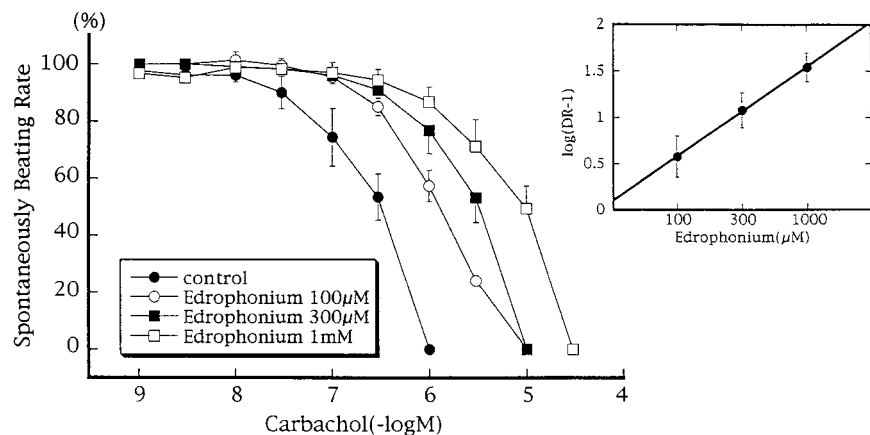


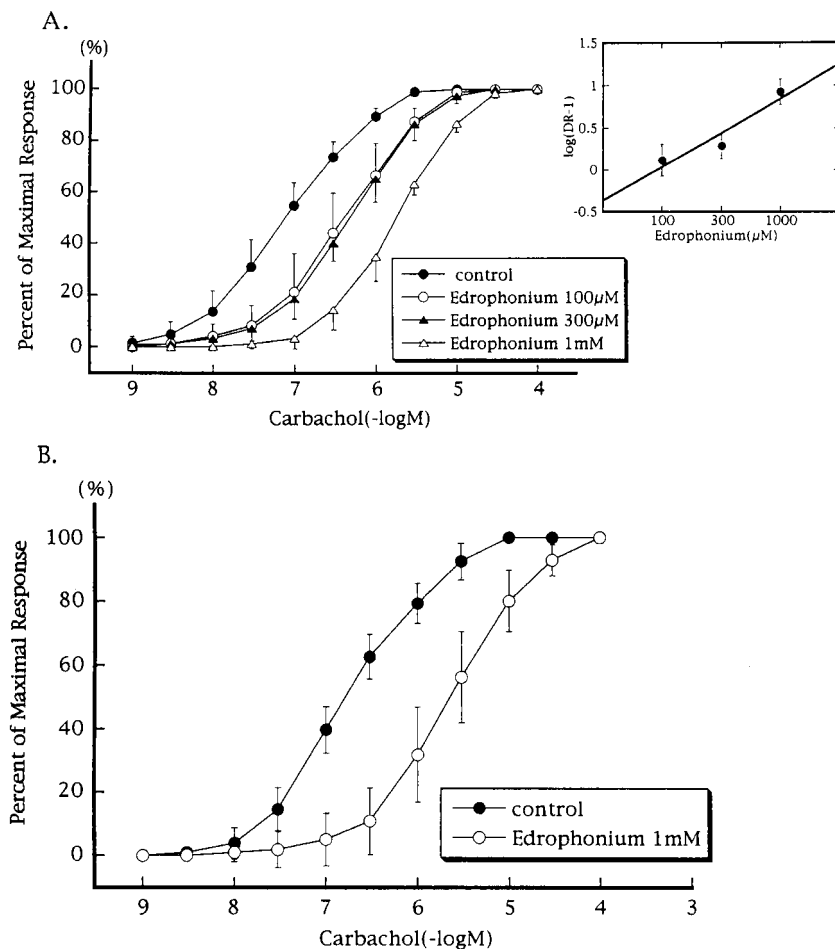
Fig. 1. Concentration-response curves for the negative chronotropic effect of carbachol in the spontaneously beating guinea pig right atria in the absence (closed circles,  $n = 17$ ) and presence of edrophonium at concentrations of 100  $\mu\text{M}$  (open circles,  $n = 4$ ), 300  $\mu\text{M}$  (closed squares,  $n = 5$ ), and 1 mM (open squares,  $n = 8$ ). Points are mean  $\pm$  SD. Pre-drug control beating rate was taken as 100%. (Inset) Schild plot of edrophonium.

carbachol in the absence of edrophonium and in its presence at concentrations of 100  $\mu\text{M}$ , 300  $\mu\text{M}$ , and 1 mM were  $92.4 \pm 36.6$  nM ( $n = 18$ ),  $209.3 \pm 101.8$  nM ( $n = 7$ ),  $254.0 \pm 62.7$  nM ( $n = 7$ ), and  $876.1 \pm 340.8$  nM ( $n = 8$ ), respectively. The slope of the regression line ( $0.84 \pm 0.25$ ) obtained from the Schild plot was not significantly different from unity. The  $\text{pA}_2$  value of edrophonium was 4.03. Edrophonium (1 mM) also shifted the concentration–response curve for the contractile effect of carbachol on human bronchial smooth muscle to the right in a parallel manner (fig. 2B). The  $\text{EC}_{50}$  values of carbachol in the human bronchus in the absence and presence of 1 mM edrophonium were  $0.19 \pm 0.05$   $\mu\text{M}$  and  $2.66 \pm 1.34$   $\mu\text{M}$ , respectively ( $n = 9$ ). The  $\text{pK}_B$  value for edrophonium was  $3.85 \pm 0.28$ .

#### Effect of Edrophonium on Muscarinic Acetylcholine Receptor-operated $\text{K}^+$ Current in Guinea Pig Atrial Cells

As shown in figure 3A, carbachol at a concentration of 1  $\mu\text{M}$  activated an outward  $\text{K}^+$  current rapidly in the atrial cells held at  $-50$  mV. After the activation, the carbachol-induced  $\text{K}^+$  current gradually declined, possibly because of a desensitization.<sup>29</sup> It has been demonstrated that intracellular application of the nonhydrolyz-

able GTP analog can directly activate GTP-binding proteins and evoke persistent activation of  $\text{I}_{\text{K,ACh}}$ .<sup>29,30</sup> Consistently, when a GTP $\gamma$ S (100  $\mu\text{M}$ )-loaded pipette was used, an outward  $\text{K}^+$  current was activated gradually after the break of the patch membrane even in the absence of any agonists (fig. 3B). It has also been shown that  $\text{A}_1$  adenosine receptor stimulation can induce  $\text{I}_{\text{K,ACh}}$  through the activation of pertussis toxin-sensitive GTP binding protein, which  $\text{A}_1$  adenosine receptors share with  $\text{M}_2$  muscarinic receptors in atrial cells.<sup>31</sup> Figure 3C shows the typical outward  $\text{K}^+$  current induced by 10  $\mu\text{M}$  adenosine in the GTP-loaded atrial cell. The peak amplitudes of the carbachol-, GTP $\gamma$ S-, and adenosine-induced outward currents were  $12.3 \pm 2.4$  (n = 7),  $7.4 \pm 0.8$  (n = 5), and  $3.7 \pm 0.5$  pA/pF (n = 7), respectively. We examined the effects of edrophonium on  $\text{I}_{\text{K,ACh}}$  evoked in these three different ways. Edrophonium at a concentration of 1 mM abolished the carbachol-induced outward  $\text{K}^+$  current, and the outward current reappeared on washout of edrophonium (fig. 3A). In seven atrial cells, edrophonium decreased the carbachol-induced current by  $98.5 \pm 1.5\%$ . On the other hand, edrophonium at the same concentration did not inhibit both GTP $\gamma$ S- and adenosine-induced outward currents (figs. 3B and 3C). In four other atrial cells in the GTP $\gamma$ S group and six



**Fig. 2.** Effects of edrophonium on the contractile response of airway smooth muscle to carbachol. (A) Concentration–response curves for the contractile effect of carbachol in the guinea pig trachea in the absence (closed circles,  $n = 18$ ) and presence of 100  $\mu\text{M}$  (open circles,  $n = 7$ ), 300  $\mu\text{M}$  (closed triangles,  $n = 7$ ), and 1 mM edrophonium (open triangles,  $n = 8$ ). Points are mean  $\pm$  SD. (Inset) Schild plot of edrophonium. (B) Concentration–response curves for the contractile effect of carbachol in the human bronchus in the absence (closed circles) and presence of edrophonium at a concentration of 1 mM (open circles). Points are mean  $\pm$  SD ( $n = 9$ ).

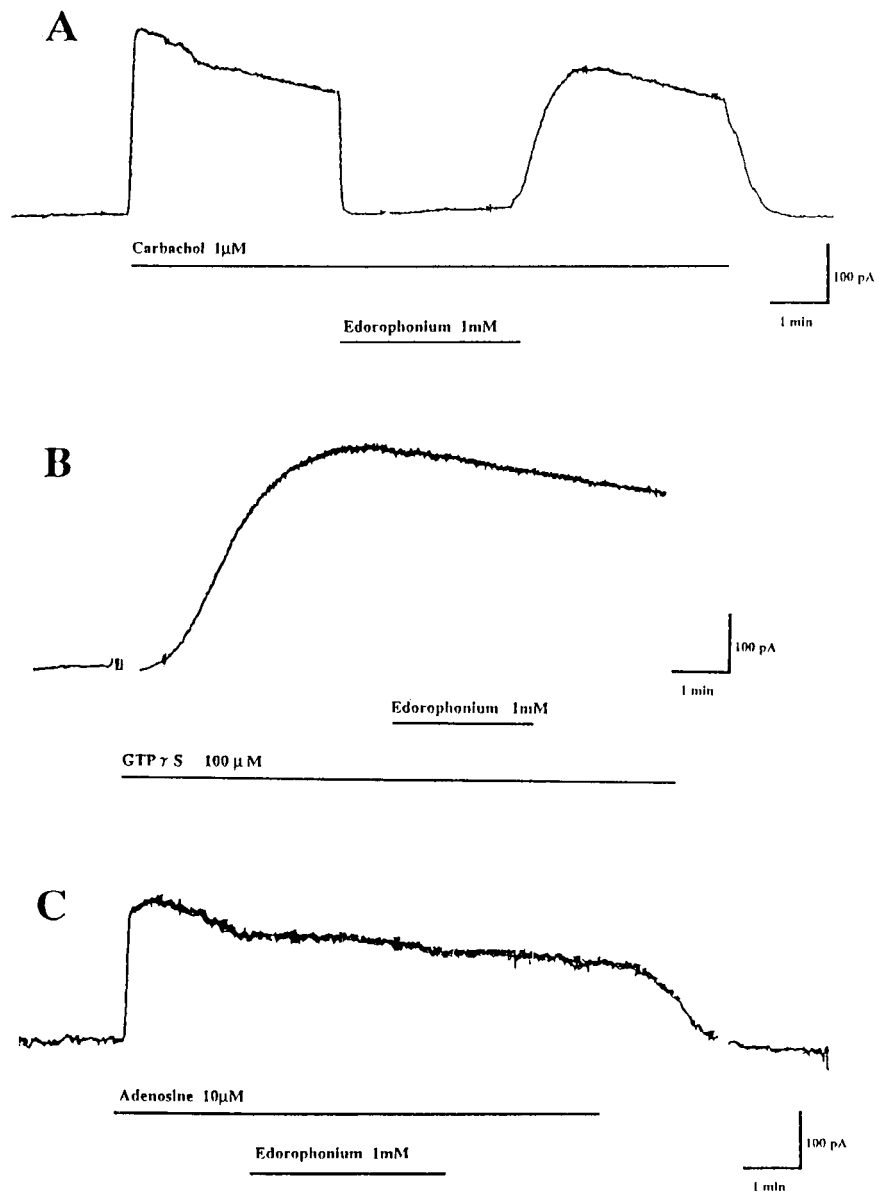


Fig. 3. Representative current tracings showing effects of edrophonium on the muscarinic acetylcholine receptor-operated  $K^+$  current activated by the extracellular application of carbachol (A), the muscarinic acetylcholine receptor-operated  $K^+$  current activated by intracellular loading of guanosine triphosphate (GTP) $\gamma$ S (B), and the adenosine receptor-operated  $K^+$  current activated by the extracellular application of adenosine (C). In (A) and (C), GTP (100  $\mu$ M) was added to the pipette solution. The holding potential was  $-50$  mV.

other cells in the adenosine group, we confirmed the negligible effect of edrophonium.

#### *Binding Profile of Edrophonium to Atrial and Glandular Muscarinic Acetylcholine Receptors*

Figure 4 shows the representative saturation isotherm for [ $^3$ H]NMS and its Scatchard plot in guinea pig atrial membranes. It showed that the radioligand interacted with a single population of saturable high-affinity sites with a  $K_d$  of 160 pM and a  $B_{max}$  of 246.8 fmol/mg protein. As shown in figure 5, [ $^3$ H]NMS also interacted with a single population of saturable high-affinity sites in guinea pig SG membranes with a  $K_d$  of 81.9 pM and  $B_{max}$  of 97 fmol/mg protein. Table 1 summarizes  $K_d$  and  $B_{max}$  values obtained from three individual experiments in the atrial and SG membranes. The  $K_d$  value for [ $^3$ H]NMS at the cardiac muscarinic receptors was approximately twofold greater than

that at the glandular receptors, whereas  $B_{max}$  of the former was also twofold greater than that of the latter, as previously reported.<sup>32</sup> The representative Scatchard plots obtained from the saturation binding experiments in the presence of edrophonium are shown in figures 4B and 5B, and the  $K_d$  and  $B_{max}$  in the presence of the agent are summarized in table 1. Edrophonium at concentrations of 30 and 100  $\mu$ M significantly decreased the affinity of [ $^3$ H]NMS for the binding sites without affecting  $B_{max}$  in both the atrial and SG membrane preparations.

Edrophonium inhibited the specific 200-pM [ $^3$ H]NMS binding in the atrial and the SG membrane in a concentration-dependent manner (figs. 6A and 6B) with pseudo-Hill coefficient values of  $1.02 \pm 0.03$  and  $1.07 \pm 0.06$  ( $n = 3$  for each), respectively, suggesting that edrophonium interacted with homogenous population of binding sites in each membrane preparation.  $K_i$  values of

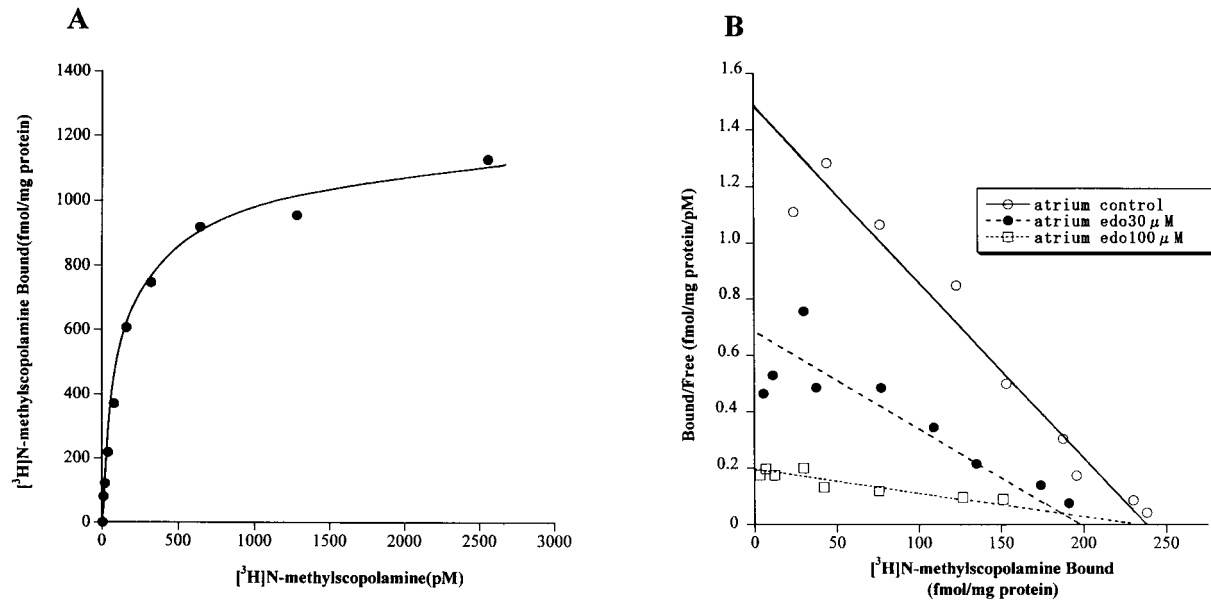


Fig. 4. Representative saturation isotherm for  $[^3\text{H}]$ N-methylscopolamine binding to membrane prepared from guinea pig left atrium (A) and Scatchard plots of the data in the absence (control) and presence of edrophonium at concentrations of 30 and 100  $\mu\text{M}$  (B).

edrophonium for muscarinic receptors in the atrial and SG membrane preparations were  $21 \pm 5$  and  $34 \pm 6$   $\mu\text{M}$ , respectively. The affinity for the cardiac muscarinic receptors was slightly but significantly higher than the glandular muscarinic receptors ( $P = 0.026$ ). An increase in the concentration of  $[^3\text{H}]$ NMS to 500 pM shifted rightward the displacement curve for edrophonium in the atrial membrane preparations with a pseudo-Hill coefficient value of  $1.00 \pm 0.06$  ( $n = 3$ ); however, the calculated  $K_i$  value of  $26.2 \pm 5.8$   $\mu\text{M}$  was not significantly different from the  $K_i$  value obtained from the experi-

ments using 200 pM  $[^3\text{H}]$ NMS. Similarly, in the SG membrane preparations, the  $K_i$  value of edrophonium obtained from the displacement curve using 500 pM  $[^3\text{H}]$ NMS (28.8  $\mu\text{M}$ ) was close to the  $K_i$  value obtained from the experiments using 200 pM  $[^3\text{H}]$ NMS.

In the atrial membrane preparations, 200 pM  $[^3\text{H}]$ NMS dissociated from the specific binding sites almost completely 20 min after the addition of 1  $\mu\text{M}$  atropine, as shown previously.<sup>23</sup> Edrophonium at concentrations up to 1 mM did not disturb the atropine-induced dissociation of  $[^3\text{H}]$ NMS, whereas the drug in concentrations higher

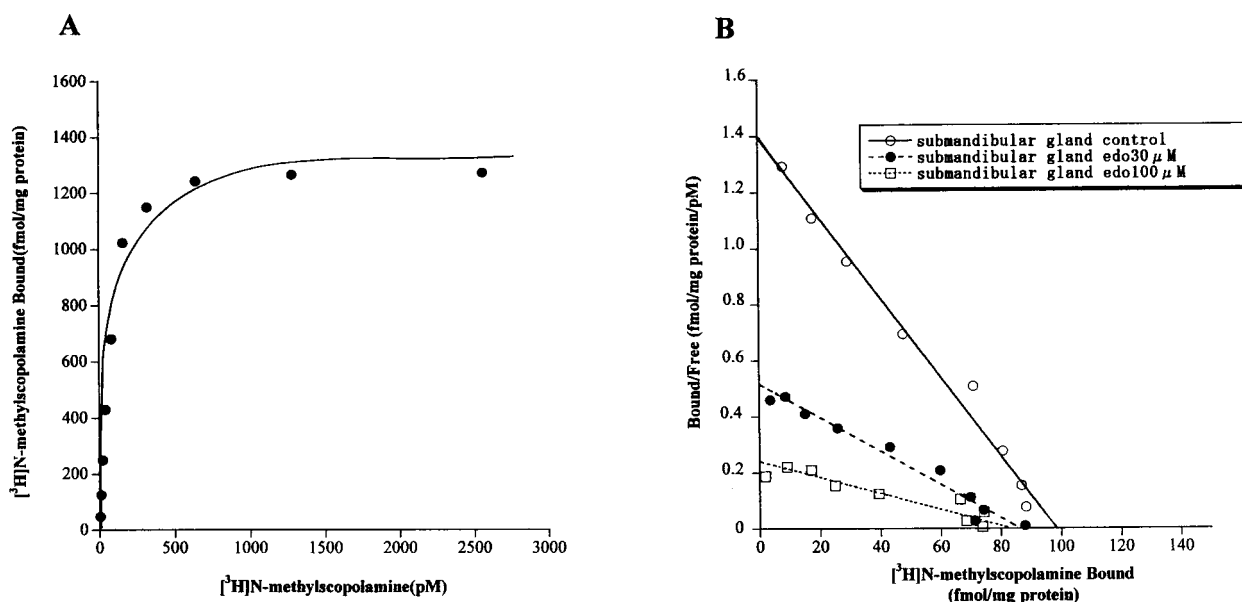


Fig. 5. Representative saturation isotherm for  $[^3\text{H}]$ N-methylscopolamine binding to membrane prepared from guinea pig submandibular gland (A) and Scatchard plots of the data in the absence (control) and presence of edrophonium at concentrations of 30 and 100  $\mu\text{M}$  (B).

**Table 1. Equilibrium Dissociation Constant ( $K_d$ ) and Maximum Binding Capacity ( $B_{max}$ ) for the Binding of [ $^3$ H]NMS to Cardiac and Submandibular Muscarinic Receptors in the Absence and Presence of Edrophonium**

	Cardiac (n = 3)		Submandibular gland (n = 3)	
	$K_d$ (pM)	$B_{max}$ (fmol/mg protein)	$K_d$ (pM)	$B_{max}$ (fmol/mg protein)
Control	201 ± 49	240.4 ± 73.8	73 ± 17	80.6 ± 14.8
Edrophonium				
30 μM	674 ± 277*	241.6 ± 4.2	194 ± 44*	90.0 ± 7.9
100 μM	1,045 ± 244†	239.2 ± 38.1	442 ± 85†	86.8 ± 6.4

Values are mean ± SD.

\*  $P < 0.05$ , †  $P < 0.01$ , significantly different from corresponding control.

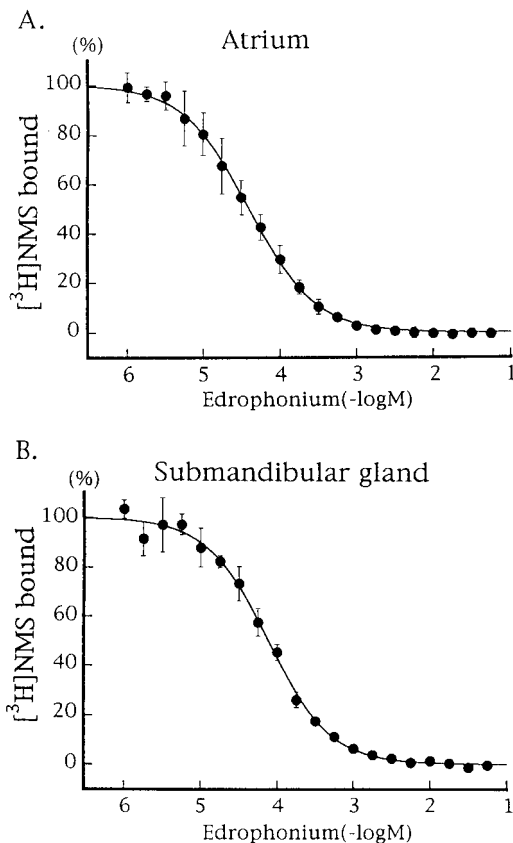
[ $^3$ H]NMS = [ $^3$ H]N-methyl-scopolamine.

than 3 mM significantly inhibited it (n = 3, data not shown). In addition to the cardiac preparations, we confirmed that high concentrations of edrophonium inhibited the atropine-induced dissociation of the radioligand in the SG membrane preparations (n = 2, data not shown).

## Discussion

In the current study, edrophonium antagonized the carbachol-induced decrease in the spontaneously beating rate of guinea pig right atria and the carbachol-induced increase in the force of contraction of guinea pig tracheal smooth muscles in a competitive manner. The drug inhibited the carbachol-induced outward current but not the adenosine- or GTPγ S-induced current. Consistent with the functional experiments, edrophonium inhibited the specific [ $^3$ H]NMS binding to membrane preparations obtained from guinea pig atria and SGs. The affinities of edrophonium for the peripheral muscarinic receptors obtained from the functional experiments were similar to those obtained from the radioligand binding studies. These results indicate that edrophonium directly interacts with peripheral muscarinic receptors and functions as a competitive antagonist.

Edrophonium shifted the concentration-response curve for the negative chronotropic effect of carbachol in a concentration-dependent manner without affecting the maximal effect of carbachol. The slope of the Schild plot (0.96) was not different from unity, indicating that edrophonium blocked the cardiac muscarinic receptor competitively. Theoretically, edrophonium can inhibit the negative chronotropic effect of carbachol in at least three different ways. The signal transduction pathway that mediates the negative chronotropic effect of carbachol is composed of the muscarinic receptors, the inhibitory GTP-binding proteins (Gi), and the mAChR-operated  $K^+$  channels ( $K_{ACh}$  channels).<sup>33</sup> Therefore, there are three possible mechanisms by which edrophonium inhibits the negative chronotropic effect of carbachol: (1) inhibition of the interaction of the carbachol with the  $M_2$  receptors, (2) inhibition of Gi, and (3) inhibition of  $K_{ACh}$  channels. To clarify the precise mechanism of the inhibitory effect of edrophonium, we examined the effect of the drug on the  $I_{K_{ACh}}$  evoked by carbachol, GTPγ S, or adenosine. GTPγ S activates Gi directly, and adenosine activates  $K_{ACh}$  channels through the  $A_1$  adenosine receptor-Gi pathway.<sup>29-31</sup> As demonstrated in figure 3,



**Fig. 6. Competition for [ $^3$ H]N-methyl-scopolamine ([ $^3$ H]NMS) binding by edrophonium in membrane preparations of guinea pig atria (A) and submandibular glands (SGs) (B). The concentration of [ $^3$ H]NMS was 200 pM. Data are the mean ± SD of three independent experiments performed in triplicate and are presented as the percentage of specific binding obtained in the absence of edrophonium.**



edrophonium only inhibited the current evoked by carbachol, indicating that edrophonium interacts with the cardiac mAChR without inhibiting Gi or  $K_{ACh}$  channels.

Edrophonium antagonized the contractile effect of carbachol in the tracheal smooth muscle (fig. 1B). Recent studies<sup>14,15</sup> showed that the  $M_2$  receptor stimulation can increase the force of contraction of smooth muscles indirectly by decreasing the intracellular cyclic adenosine monophosphate content when a cyclic adenosine monophosphate-generating agent relaxes the smooth muscles. However, it is acknowledged that the receptor subtype that mediates the direct contractile effect of muscarinic agonists in tracheal muscles is  $M_3$ .<sup>13,15</sup> Inasmuch as we did not use a compound that increases an intracellular cyclic adenosine monophosphate content, only  $M_3$  receptors are thought to be responsible for the contractile effect of carbachol in the current study. Therefore, it is likely that the inhibitory effect of edrophonium derived from the interaction of the drug with  $M_3$  receptors. Schild plot analysis indicated the competitive antagonism of the drug with a  $pA_2$  value of 4.03 (93.3  $\mu M$ ). These functional experiments strongly suggested that edrophonium inhibits the mAChR-mediated responses by means of a competitive antagonism against  $M_2$  and  $M_3$  muscarinic receptors.

The radioligand binding experiments were performed to further test this possibility. Recent studies showed the coexistence of  $M_1$  and  $M_2$  mAChRs in rat and guinea pig ventricular myocytes.<sup>9,10</sup> However, Northern blot analysis could only detect  $m_2$  mAChR mRNA in both cardiac atrial and ventricular cells.<sup>9,11</sup> Reverse-transcriptase polymerase chain reaction method was required to detect  $m_1$  mAChR mRNA in the ventricular cells,<sup>9,10</sup> indicating that the amount of  $M_2$  mAChR is much higher than that of  $M_1$  mAChR in the heart.<sup>9</sup> Therefore, the results obtained from the radioligand binding experiments using the atrial membrane preparations in the current study are thought to reflect the interaction of edrophonium with the  $M_2$  receptors. On the other hand, there is no direct evidence that the mAChR subtype existing in guinea pig submandibular gland is solely  $M_3$ , although mAChR subtype in rat salivary gland is thought to be  $M_3$ .<sup>11,34,35</sup> However, several pieces of evidence described below suggest that almost all guinea pig SG mAChR consists of  $M_3$  subtype. Moriya *et al.*<sup>34</sup> reported that the  $K_d$  of NMS (165 nM) for human  $M_2$  mAChR expressed in Sf9 insect cell is approximately twofold greater than that for human  $M_3$  mAChR (99 nM). Consistently, the  $K_d$  of NMS for the cardiac mAChR ( $M_2$  subtype) was approximately twofold greater than that for SG mAChR in the current study. In addition, the time course of dissociation of [<sup>3</sup>H]NMS from the guinea pig SG membrane preparations was well fitted by single-exponential function with a time constant of 72 min,<sup>32</sup> which is consistent with the report by Michel *et al.*<sup>36</sup> using the rat salivary gland.

As shown in figure 6A, edrophonium at concentrations

up to 1 mM competed with 200 pM [<sup>3</sup>H]NMS for cardiac muscarinic receptors. The pseudo-Hill coefficient value of 1.02 suggested the interaction of edrophonium with the homogenous population of binding sites. In the SG membrane preparations, edrophonium also inhibited the specific binding of 200 pM [<sup>3</sup>H]NMS in a concentration-dependent manner with a pseudo-Hill coefficient value of 1.07 (fig. 6B). A dissociation kinetic study is often performed to test the existence of allosteric interaction of an agent with the muscarinic receptors.<sup>27</sup> Atropine, a well-known competitive antagonist against the muscarinic receptors, at a concentration of 1  $\mu M$  rapidly dissociated 200 pM [<sup>3</sup>H]NMS from the cardiac muscarinic receptors with a time constant of 5.0 min.<sup>32</sup> Higher concentrations of edrophonium inhibited the atropine-induced dissociation, suggesting that edrophonium in concentrations higher than 3 mM interacts with the cardiac muscarinic receptors in an allosteric manner. Edrophonium in similar concentrations also interfered the atropine-induced dissociation of [<sup>3</sup>H]NMS from the SG membrane preparation. In other words, edrophonium in concentrations up to 1 mM would hardly bind to the allosteric site of the muscarinic receptors. Supporting this hypothesis, edrophonium in concentrations of 30 and 100  $\mu M$  increased the  $K_d$  values of NMS for the muscarinic receptors without changing  $B_{max}$  in both the cardiac and SG membrane preparations (figs. 4 and 5 and table 1). Furthermore, the  $K_i$  values of edrophonium were not changed by an increase in the concentration of [<sup>3</sup>H]NMS from 200 to 500 pM in both the membrane preparations. Taken together, these results strongly suggest that edrophonium in concentrations up to 1 mM competitively interacts with mAChRs.

The  $K_i$  value of edrophonium for the cardiac muscarinic receptors was almost equal to the affinity of edrophonium for the cardiac mAChR obtained from the functional study (24.5  $\mu M$ ). On the other hand, the  $pA_2$  value of edrophonium for the tracheal smooth muscle mAChR obtained from the functional study (4.03, *i.e.*, 93.3  $\mu M$ ) is slightly higher than the  $K_i$  value (34  $\mu M$ ) of the agent for the SG membrane mAChR calculated from the binding study. A  $pA_2$  value obtained from functional experiments is not necessarily equal to a  $K_d$  obtained from radioligand binding experiments. However, considering that the  $pA_2$  value is equal to the  $K_d$  in the heart, edrophonium might possess other effects and interfere with the precise evaluation of the antagonistic potency of edrophonium in the smooth muscle preparations. As previously mentioned, edrophonium could increase a basal tension of the tracheal smooth muscle in an atropine-sensitive manner. Because the  $K_i$  of edrophonium for the  $M_3$  receptors (34  $\mu M$ ) is almost equal to that for the  $M_2$  receptors (21  $\mu M$ ), edrophonium appears to be a nonselective antagonist against  $M_2$  and  $M_3$  muscarinic receptors.

Edrophonium also shifted the concentration-response curve for the contractile effect of carbachol on the human broncheal smooth muscle, of which contraction is mediated *via*  $M_3$ .<sup>14</sup> The  $pK_B$  value, calculated from the experiments using 1 mM edrophonium, was similar with the  $pA_2$  value of edrophonium for the guinea pig tracheal smooth muscle. Therefore, it may be possible to extrapolate the current findings obtained from guinea pigs to humans.

It is interesting to note that the bradycardic effect of edrophonium is clinically less potent than that of neostigmine when muscle weakness produced by nondepolarizing muscle relaxants is reversed by the acetylcholinesterase inhibitors.<sup>4</sup> In this regard, Backman *et al.*<sup>5</sup> demonstrated that the bradycardic effect of edrophonium in anesthetized cats was well correlated with its inhibition of acetylcholinesterase activity, whereas the bradycardia induced by neostigmine was greater than that expected from the inhibitory effect on the acetylcholinesterase activity. The investigators suggested that neostigmine not only inhibited acetylcholinesterase but also activated mAChR within the cardiac parasympathetic pathway directly. Consistently, we have recently demonstrated that neostigmine, but not edrophonium, at clinically relevant concentrations decreased the spontaneously beating rate of isolated guinea pig right atria.<sup>6</sup> In addition to lack of the direct negative chronotropic effect, the antimuscarinic effect of edrophonium, shown in the current study, could also contribute to the less potent bradycardic effect. Although the affinity of edrophonium for the muscarinic receptors seems to be approximately 10–20 times lower than the inhibition constant of the drug for acetylcholinesterase (1.5–6.5  $\mu M$ ),<sup>37,38</sup> a plasma peak level of 50  $\mu M$  is expected after administration of edrophonium at a dose of 1 mg/kg.<sup>39</sup> This concentration seems to be sufficient for edrophonium to produce an antimuscarinic effect against both  $M_2$  and  $M_3$  muscarinic receptors.

In conclusion, the acetylcholinesterase inhibitor edrophonium inhibits the negative chronotropic effect and bronchoconstricting effect of carbachol in a competitive manner. The radioligand binding experiments showed that edrophonium binds primarily to a competitive antagonist-binding site of the  $M_2$  and  $M_3$  receptors with almost equal affinities. However, in higher concentrations, the drug may also interact with the receptors allosterically. The comparison between the affinities of edrophonium obtained from the functional and binding experiments suggested the pharmacologic significance of the interaction of the drug with the primary binding site. Edrophonium, when administered for reversing muscle weakness produced by a nondepolarizing neuromuscular blocking drug, can reach a plasma concentration sufficient to produce the antimuscarinic effects.

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