A Difference in Receptor Mechanisms for Muscarinic Full and Partial Agonists

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ABSTRACT — Concentration-response curves of 4 muscarinic full agonists were progressively inhibited by 10 to 50-min treatments of the longitudinal muscle of guinea pig ileum with propylbenzilylcholine mustard (PrBCM, 3 × 10⁻⁶ M). A 90-min treatment with PrBCM had no further significant inhibitory effect on their curves. The 50-min treatment with PrBCM (3 × 10⁻⁶ M) completely inhibited the concentration-response curves of 6 partial agonists. The limiting effect of PrBCM observed on the concentration-response curves of the full agonists was not found on the curves of the partial agonists. These results suggest that there are two subtypes of M₃-cholinoceptors, PrBCM-sensitive receptors and PrBCM-resistant ones. Pilocarpine, a partial agonist, shifted the concentration-response curve of carbachol, a full agonist, in a parallel fashion in the strips treated with PrBCM (3 × 10⁻⁶ M) for 50 min, suggesting that an interaction of pilocarpine with PrBCM-resistant cholinoceptors does not induce contraction. The full agonists contract the longitudinal muscle through the interaction of two cholinoceptors, PrBCM-sensitive and -resistant ones, while the partial agonists produce the contraction through the activation of PrBCM-sensitive ones.

There is now considerable evidence that muscarinic cholinoceptors can be divided into two subtypes: M₁-receptors with a high affinity and M₂-receptors with a low affinity for pirenzepine (1–3). Binding sites with low affinities for pirenzepine are not a homogeneous population and can be further subdivided into two groups. AF-DX 116 [11-(((dimethylamino)methyl)-1-piperidinyl)acetyl]-5,11-dihydro-6H-pyrido(2,3-b)(1,4)benzodiazepine-6-one] binds with a high affinity for muscarinic binding sites in the heart and other tissues, whereas 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiobromide) has a higher affinity for muscarinic binding sites in exocrine glands than for those of the heart (4). Doods et al. (5) classified muscarinic cholinoceptors with a low affinity for pirenzepine and a high affinity for AF-DX 116 as M₂-receptors and ones with a low affinity for pirenzepine and a high affinity for 4-DAMP as M₃-receptors. In our laboratory (6, 7), two different subtypes of M₃-receptors were identified in the taenia caecum of guinea pigs using propylbenzilylcholine mustard (PrBCM), an irreversible specific muscarinic antagonist (8): PrBCM-sensitive and -resistant receptors. In this paper we tried to characterize these two muscarinic cholinoceptor mechanisms using the longitudinal muscle of guinea pig ileum.

MATERIALS AND METHODS

Male guinea pigs, weighing 300 to 400 g, were killed by a blow on the head. A longitudinal muscle strip was isolated by carefully
slipping an ileal segment over a tapered glass rod. A piece (about 3 cm) of strip was suspended in a 20-ml organ bath filled with a physiological solution, of the following composition: 154 mM NaCl, 5.6 mM KCl, 2.1 mM CaCl₂, 0.8 mM MgCl₂, 6.0 mM NaHCO₃ and 2.8 mM glucose, kept at 37°C and gassed with a mixture of 95% O₂ and 5% CO₂. Responses to agonists were isotonically recorded with a load of 0.7 g. Concentration-response curves of agonists were obtained cumulatively in the presence of tetrodotoxin (3 × 10⁻⁷ M).

For occlusion of muscarinic cholinoreceptors, after the control concentration-response curves of agonists were determined, the strip was treated for 5, 10, 20, 50 and 90 min with PrBCM (3 × 10⁻⁶ M) according to the method of Hisayama et al. (6). When PrBCM was applied for longer than 10 min, it was renewed every 10 min. The strip was then allowed to re-equilibrate for 30 min with washing every 10 min. The concentration-response curves for the agonists were determined after repeating determinations of the curves for carbachol until constant ones were established. In some experiments, phenoxybenzamine (3 × 10⁻⁶ M) was used as an irreversible cholinoreceptor blocker. To estimate the dissociation constant, Kᵦ, of carbachol or pilocarpine, the strips were treated with phenoxybenzamine, according to Furchgott (9). Procedures followed almost the same protocol as in the experiments with PrBCM. The dissociation constants of carbachol and pilocarpine were obtained from the equation (i):

$$\frac{1}{[A]} = \frac{1}{q[A']} + \frac{1-q}{qK_{A}}$$  \hspace{1cm} (i)

where [A] and [A'] are the corresponding equieffective concentrations of carbachol and pilocarpine before and after irreversible blockade of a fraction of the receptors, and q is the remaining fraction of active receptors after irreversible blockade. If 1/[A] is plotted versus 1/[A'] and a straight line fitted to the data by linear regression analysis, the dissociation constant, Kᵦ, can be calculated by the following equation (ii):

$$K_{A} = \frac{\text{slope} - 1}{\text{intercept of Y-axis}}$$  \hspace{1cm} (ii)

To estimate the dissociation constant of a competitive antagonist, the pA₂ value (a negative logarithm of the dissociation constant) can be calculated by the method of Arunlakshana and Schild (10) as modified by Tallarida et al. (11). After determining the control concentration-response curve for an agonist, the strip was equilibrated with a competitive antagonist for 30 min. A concentration-response curve for agonist was then obtained in the presence of the antagonist, and the procedure repeated with a high (either 3-fold or 10-fold) concentration in the same strip. The pA₂ value was calculated from parallel shifts of the curve for the agonist.

To stimulate cholinergic nerves in the longitudinal muscle, a 20-ml organ bath was used, where two platinum electrodes (2 × 45 mm) were set about 7 mm apart, containing the physiological solution gassed with a mixture of 95% O₂ and 5% CO₂ and kept at 37°C. A piece (about 3 cm) of the strip was placed between the two electrodes. Field stimulation was carried out by passing rectangular pulses of 0.5 msec, supramaximal voltage and frequency of 0.1 Hz between the two electrodes. Response to electrical stimulation was recorded isometrically with an initial tension of 0.7 g.

The data are expressed as means with S.E., and Duncan's new multiple range test was used to calculate statistical significance where appropriate. A P value less than 0.05 was considered a significant difference.

Drugs used: carbachol chloride (Sigma), pilocarpine hydrochloride (Sigma), atropine sulfate (Sigma), nicotine bitartrate (Nacalai Tesque), propylbenzilylcholine mustard hydrochloride (New England Nuclear), phenoxybenzamine hydrochloride (Tokyo Kasei), 4-DAMP (Research Biochemicals Inc.) and tetrodotoxin (Sankyo). Butyltrimethylammonium bromide, hexyltrimethylammonium bromide, heptyltrimethylammonium bromide, propionylcholine iodide, butyrylcholine iodide, pen-
tanoylcholine iodide, hexanoylcholine iodide and benzoylcholine iodide were synthesized in our laboratory. Other chemicals used were of analytical grade.

RESULTS

Concentration-response curves of carbachol, butyltrimethylammonium, pilocarpine and benzoylcholine were shifted to the right in a parallel manner by the 30-min treatment with 4-DAMP. Schild plots of these results yielded straight line with a slope of 1. The pA2 values of 4-DAMP estimated by Schild plot analysis were $8.15 \pm 0.04$ $(N = 5)$ against carbachol, $8.09 \pm 0.07$ $(N = 5)$ against butyltrimethylammonium, $8.35 \pm 0.03$ $(N = 5)$ against pilocarpine and $8.30 \pm 0.05$ $(N = 5)$ against benzoylcholine. These values are in good agreement with that reported by Doods et al. (5), suggesting that the agonists used herein interact with M3-cholinoreceptors as reported previously (12).

Carbachol, butyltrimethylammonium (Fig. 1), propionylcholine and butyrylcholine behaved as full agonists on M3-receptors in the longitudinal muscle of guinea pig ileum. They were progressively inhibited by the 10 to 50-min treatment with PrBCM $(3 \times 10^{-6} \text{M})$. As the results were in good agreement with those of Hisayama et al. (6) and Takayanagi et al. (7) in the guinea pig taenia caecum, the curves after the 10- and 20-min treatments were not shown in Fig. 1. The 90-min treatment with PrBCM had no further significant inhibitory effect on their concentration-response curves (Fig. 1).

In the strips treated with PrBCM $(3 \times 10^{-6} \text{M})$ for 50 min, the concentration-response curves for carbachol and butyltrimethylammonium were shifted to the right in a parallel manner by 4-DAMP. Schild plots of these re-

![Fig. 1](image.png)

Fig. 1. Irreversible inhibitory effects of 50 and 90 min treatments with PrBCM $(3 \times 10^{-6} \text{M})$ on concentration-response curves of full agonists, carbachol (A) and butyltrimethylammonium (B). Ordinate: contraction (%) which is expressed as a percent of the contractile response to carbachol $(10^{-6} \text{M})$ and abscissa: logarithm of drug concentration $(\text{M})$. Each value is expressed as a mean ± S.E. (bar) of 7 experiments. (A): •, carbachol before treatment; (B): ⊗, carbachol as a reference agonist; ○, butyltrimethylammonium before treatment; ▲, after a 50-min treatment; △, after a 90-min treatment.
results yielded straight lines with a slope of 1. The pA\textsubscript{2} values for 4-DAMP were 8.31 ± 0.05 (N = 5) against carbachol and 8.44 ± 0.10 (N = 5) against butyltrimethylammonium. These values are in good agreement with those obtained in the untreated strips as reported previously (12).

Pilocarpine, heptyltrimethylammonium, benzoylcholine (Fig. 2), hexyltrimethylammonium, pentanoylcholine and hexanoylcholine acted as partial agonists. The concentration-response curves of these partial agonists were strongly inhibited by the 50-min treatment. The ratios of the maximum contractions induced by the agonists before and after the treatment of the strips with PrBCM against that by carbachol, a reference agonist, before the treatment are summarized in Fig. 3. The maximum contractions by the partial agonists after the PrBCM treatment were not significantly different from

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Fig. 2. Irreversible inhibitory effects of 50- and 90-min treatments with PrBCM (3 × 10^{-6} M) on concentration-response curves of partial agonists, pilocarpine (A), benzoylcholine (B) and heptyltrimethylammonium (C). Ordinate: contraction (%) which is expressed as a percent of the contractile response to carbachol (10^{-6} M) and abscissa: logarithm of drug concentration (M). Each value is expressed as a mean ± S.E. (bar) of 7 experiments. ●, carbachol as a reference agonist; ○, before treatment; ▲, after a 50-min treatment; △, after a 90-min treatment.
zero. The efficacies for the agonists used here-
in were estimated by Stephenson (13) and Takayanagi et al. (14). The agonists with re-
latively larger efficacies tended to induce the larger maximum contractions in the PrBCM-
treated strips (Fig. 3). In the strips treated with PrBCM (3 \times 10^{-6} \text{ M}) for 50 min, where the partial agonists did not contract, the concentration-response curve of carbachol was shifted to the higher concentration in a parallel manner by the 10-min treatment with pilo-
carpine (10^{-5}, 3 \times 10^{-5} \text{ and } 10^{-4} \text{ M}) (Fig. 4). The Schild plot of these results yielded a straight line with a slope of 1. The pA\textsubscript{2} value of pilocarpine estimated by Schild plot analysis was 5.42 ± 0.04. The dissociation constant, pK\textsubscript{A} value of pilocarpine, was determined by the method of Furchgott (9) using phenoxy-
benzamine to occlude a fraction of the M\textsubscript{3} receptors. The concentration-response curve of pilocarpine was inhibited by the 3-min treatment with phenoxybenzamine (3 \times 10^{-6} \text{ M}) (Fig. 5). The pK\textsubscript{A} value estimated by the method of Furchgott (9) was 4.86 ± 0.15.

The dissociation constant of carbachol be-
fore and after the PrBCM treatment was also estimated by the same method. The control concentration-response curves of carbachol in the strips untreated and treated with PrBCM

![Fig. 3](image-url)  
**Fig. 3.** Relation between the maximum contractions before and after a 50-min treatment with PrBCM (3 \times 10^{-6} \text{ M}). Ordinate: the ratio of the maximum contraction induced by a test agonist against that by car-
bachol in the untreated strips and abscissa: the ratio of the maximum contraction by a test agonist after PrBCM treatment against that by carbachol before the treatment. Each value is expressed as a mean ± S.E. of 7 experiments. (A): cholinesters, 1; hexanoylcholine, 2; benzoylcholine, 3; pentanoylcholine, 4; butyrylcholine, 5; propionylcholine, 6; carbachol. (B): alkyltrimethylammoniums and pilocarpine, 1; heptyl-
trimethylammonium, 2; hexyltrimethylammonium, 3; butyltrimethylammonium, 4; pilocarpine, 5; carbachol as a reference agonist.

![Fig. 4](image-url)  
**Fig. 4.** Inhibitory effect of pilocarpine on the concen-
tration-response curve of carbachol in the longitudinal muscle treated with PrBCM (3 \times 10^{-6} \text{ M}) for 50 min. Ordinate: contraction (%) which is expressed as a percent of the contractile response of the untreated strip to carbachol (10^{-6} \text{ M}) and abscissa: logarithm of car-
bachol concentration (M). Each value is expressed as a mean ± S.E. of 7 experiments. ●: carbachol alone; ○, △ and ▲: with pilocarpine, 10^{-5}, 3 \times 10^{-5} \text{ and } 10^{-4} \text{ M}, respectively.
(3 × 10^{-6} M) for 50 min were inhibited by the 50-min treatment with phenoxybenzamine (3 × 10^{-6} M) (Fig. 6). The pK\(_A\) values of carbachol in the untreated strips and in the strips treated with PrBCM were 4.77 ± 0.09 and 5.52 ± 0.06, respectively. The values are significantly different from each other.

Contractile responses to nicotine (10^{-5} M) and to electrical stimulation were abolished by a 15-min pretreatment of the longitudinal muscle strip with tetrodotoxin (10^{-6} M) and by a 5-min pretreatment with atropine (10^{-6} M), suggesting that the responses were induced by acetylcholine released from the cholinergic nerves (data not shown). The strips, after the 50-min treatment with PrBCM (3 × 10^{-6} M), barely responded to nicotine (Fig. 7). Contractile responses to electrical stimulation before and after the 50-min treatment with PrBCM (3 × 10^{-6} M) were inhibited by the 50-min treatment with phenoxybenzamine (3 × 10^{-6} M) (Fig. 6).

### Fig. 5
Irreversible inhibitory effect of phenoxybenzamine (3 × 10^{-6} M) on the concentration-response curve of pilocarpine in the untreated longitudinal muscle. Ordinate: contraction (%) which is expressed as a percent of the contractile response to carbachol (10^{-6} M) and abscissa: logarithm of carbachol concentration (M). Each value is expressed as a mean ± S.E. (bar) of 7 experiments. ●: carbachol as a reference agonist; ○: pilocarpine before a treatment; ▲: pilocarpine after 3-min treatment with phenoxybenzamine.

### Fig. 6
Irreversible inhibitory effect of phenoxybenzamine (3 × 10^{-6} M) on concentration-response curves of carbachol before (A) and after (B) the 50-min treatment with PrBCM (3 × 10^{-6} M). Ordinate: contraction (%) which is expressed as a percent of the contractile response to carbachol (10^{-6} M) and abscissa: logarithm of carbachol concentration (M). Each value is expressed as a mean ± S.E. (bar) of 7 experiments. (A): ●: carbachol before the 50-min treatment; ○: carbachol after 50-min treatment of the PrBCM-untreated strip with phenoxybenzamine (3 × 10^{-6} M); (B): ●: carbachol before the treatment; ○: carbachol after the PrBCM-treatment; ▲: carbachol after a 50-min treatment of the PrBCM-treated strip with phenoxybenzamine (3 × 10^{-6} M).
X 10^6 M) were, respectively, 33.9 ± 5.0% and 4.4 ± 1.4%; these values were expressed as a percent of the response to carbachol (10^-6 M), and the values are significantly different from each other.

**DISCUSSION**

The 50-min treatment of the longitudinal muscle strip with PrBCM (3 × 10^-6 M) inhibited irreversibly the concentration-response curves of the full agonists. These facts confirmed the results of Young et al. (8) who reported that PrBCM irreversibly antagonized the contractile response of the intestinal muscle to muscarinic agonists. More prolonged (90 min) treatment with PrBCM had no further significant inhibitory effect on the concentration-response curves of full agonists. The disappearance of the response to the partial agonists caused by the 50-min treatment with PrBCM suggest that they produce contraction through an activation of PrBCM-sensitive cholinoceptors. However, the fact that in the strips treated with PrBCM, pilocarpine shifted the concentration-response curve of carbachol in a parallel fashion suggest that the interaction of pilocarpine with PrBCM-resistant receptors does not induce contraction and acts as a muscarinic antagonist (Fig. 8). The pK_A value (4.77) of carbachol to PrBCM-sensitive receptors estimated in the untreated strips is significantly (P < 0.05) less than that (5.52) to PrBCM-resistant ones in the strips treated with PrBCM. Furthermore, the pK_A value (4.86) of pilocarpine is significantly (P < 0.05) less than the pA_2 value (5.42), which was estimated from an antagonism between pilocarpine and carbachol in the strips treated with PrBCM. These results are in agreement with the data obtained from receptor binding assays using membranes (15) and suggest that the affinity of cholinergic agonist to PrBCM-sensitive receptors is less than that to PrBCM-resistant ones. Recently, Takayanagi et al. (12, 16) reported that activation of PrBCM-sensitive cholinoceptors more effectively utilizes cytosolic Ca^{2+} for contraction in the longitudinal muscle of the guinea pig ileum than the activation of PrBCM-resist-

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**Fig. 7.** Irreversible inhibitory effect of the 50-min treatment with PrBCM (3 × 10^-6 M) on the contractile responses to nicotine (10^-5 M) and carbachol. Ordinate: contraction (%) which is expressed as a percent of the contractile response to carbachol (10^-6 M) and abscissa: logarithm of drug concentration (M). Each value is presented as a mean ± S.E. (bar) of 7 experiments. • and ☐: carbachol, ▲ and △: nicotine. Solid and open symbols: before and after the 50 min treatment with PrBCM (3 × 10^-6 M).

pirenzepine, AF-DX 116 and 4-DAMP (12 and the present results).

In our laboratory (15), the interrelationship between PrBCM-sensitive and -insensitive cholinoceptors was investigated using a receptor binding assay with [^3]H-quinuclidinyl benzilate (ONB) and [^3]H-PrBCM as radiolabelled ligands; and it was concluded that in the presence of guanine nucleotide (GTP), PrBCM recognizes two distinct M_3-receptors, PrBCM-sensitive receptors and PrBCM-resistant ones. These data support the results obtained in this study.

The full agonists elicit a stimulus through an interaction of two subtypes of cholinoceptors, as the limiting inhibitory effect of PrBCM was observed on the concentration-response curves of the full agonists. The disappearance of the response to the partial agonists caused by the 50-min treatment with PrBCM suggest that they produce contraction through an activation of PrBCM-sensitive cholinoceptors. However, the fact that in the strips treated with PrBCM, pilocarpine shifted the concentration-response curve of carbachol in a parallel fashion suggest that the interaction of pilocarpine with PrBCM-resistant receptors does not induce contraction and acts as a muscarinic antagonist (Fig. 8). The pK_A value (4.77) of carbachol to PrBCM-sensitive receptors estimated in the untreated strips is significantly (P < 0.05) less than that (5.52) to PrBCM-resistant ones in the strips treated with PrBCM. Furthermore, the pK_A value (4.86) of pilocarpine is significantly (P < 0.05) less than the pA_2 value (5.42), which was estimated from an antagonism between pilocarpine and carbachol in the strips treated with PrBCM. These results are in agreement with the data obtained from receptor binding assays using membranes (15) and suggest that the affinity of cholinergic agonist to PrBCM-sensitive receptors is less than that to PrBCM-resistant ones. Recently, Takayanagi et al. (12, 16) reported that activation of PrBCM-sensitive cholinoceptors more effectively utilizes cytosolic Ca^{2+} for contraction in the longitudinal muscle of the guinea pig ileum than the activation of PrBCM-resist-
ant ones. These results support the present view.

PrBCM-sensitive cholinoreceptors may play a physiological role, because the longitudinal muscle treated with PrBCM responded little to nicotine or to electrical stimulation. The results provide clues to the finding of unknown subtypes or different conformational states of M3-cholinoreceptors.

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