Characterization of Subtype of Propylbenzilylcholine Mustard (PrBCM)-Sensitive and -Resistant Muscarinic Cholinoceptors in Guinea Pig Ileal Muscle

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ABSTRACT—The subtype of propylbenzilylcholine mustard (PrBCM)-sensitive and -resistant muscarinic cholinoceptors in guinea pig ileal muscle was examined using four selective muscarinic antagonists, pirenzepine, AF-DX 116, himbacine and 4-DAMP. The pA_2 values of the four antagonists against pilocarpine were not different from their values against carbachol after the treatment with PrBCM and was identified with the values for the m_3 subtype. These results suggest that the subtype of PrBCM-sensitive and -resistant muscarinic cholinoceptors in guinea pig ileal muscle is the m_3 subtype only and not other subtypes.

Keywords: Propylbenzilylcholine mustard (PrBCM) receptor, Muscarinic m_3 subtype

Muscarinic cholinoceptors have been pharmacologically divided into three major subtypes, M_1 , M_2 and M_3 subtypes, by differences in affinities for the selective competitive antagonists (1–4). However, differing from these subclassifications based on findings obtained from functional studies using selective competitive antagonists, we have proposed that in the intestinal smooth muscles, there are two types of cholinergic mechanisms, one being sensitive to and the other being resistant to propylbenzilylcholine mustard (PrBCM), which is known to be an irreversible specific muscarinic cholinergic antagonist. Cholinergic partial agonists (e.g., pilocarpine) produce contractions of the intestinal smooth muscle through activation of PrBCM-sensitive receptors, while full agonists (e.g., carbachol), which contract it through an interaction with both types, are more effective at PrBCM-resistant receptors than at PrBCM-sensitive receptors. Pilocarpine, a partial agonist, behaves as a competitive cholinergic antagonist on PrBCM-resistant receptors in the intestinal smooth muscles treated with PrBCM (5–7). Furthermore, PrBCM-sensitive cholinoceptors were found to play a physiological role (6). However, these two types may also provide clues for finding unknown subtypes.

Recently, molecular cloning of muscarinic receptors

has demonstrated the presence of five related but distinct gene products (m_1 , m_2 , m_3 , m_4 and m_5) (8, 9). Pharmacological studies can differentiate muscarinic receptors into three types (M_1 , M_2 , M_3), and this pharmacological classification most likely corresponds to the m_1 , m_2 and m_3 receptors, respectively. More recently, Dörje et al. (10) have determined the affinity profiles of several of the selective muscarinic antagonists at five cloned human muscarinic receptors (m_1 – m_5) stably expressed in Chinese hamster ovary cells (CHO-K1).

In this paper, we tried to characterize PrBCM-sensitive and -resistant muscarinic cholinergic receptor subtypes in guinea pig ileal muscle using four selective muscarinic antagonists: pirenzepine (m_1 [M_1]-selective); AF-DX 116 (11-([2-[(diethylamino)methyl]-1-piperidinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodizepine-6-one) (m_2 [M_2]-selective); himbacine (m_2 [M_2], m_4 -selective); and 4-DAMP (4-diphenylacetoxy-N-methylpiperidine methiodide) (m_1 [M_1], m_3 [M_3]-selective).

Male guinea pigs, weighing 250 to 350 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 the 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 $CaCl_2$, 0.8 mM $MgCl_2$, 6.0 mM $NaHCO_3$ and 2.8 mM glucose, kept at 37°C and

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gassed with a mixture of 95% O₂ and 5% CO₂. Responses to agonists were isotonicity recorded under a tension of 0.7 g. Concentration-response curves of agonists were obtained cumulatively.

To exclude the effect of muscarinic cholinceptors, after the control concentration-response curve for carbachol were determined, the preparation was treated with PrBCM (3×10^{-6} M) for 50 min according to the method of Hisayama et al. (11). When PrBCM was applied for 50 min, it was renewed every 10 min. The preparation was then allowed to re-equilibrate for 30 min with washing every 10 min. The curve for carbachol was again determined after thorough washing every 10 min.

To estimate the dissociation constants of four competitive antagonists, the pA₂ value (a negative logarithm of the dissociation constant) can be calculated by the method of Arunlakshana and Schild (12) as modified by Tallarida et al. (13). After determining the control concentration-response curves for carbachol after the treatment with PrBCM (response mediated through PrBCM-resistant muscarinic cholinceptors) and pilocarpine (response mediated through PrBCM-sensitive ones), the preparation 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 preparation. The pA₂ value was calculated from parallel shifts of the curve for the agonist.

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 as a significant difference. The following drugs were used: Carbachol chloride and pilocarpine hydrochloride (Sigma, St. Louis, MO); PrBCM (New England Nuclear, Boston, MA); AF-DX 116 and himbacine (Dr. Karl Thomae GmbH, Biberach, Germany); pirenzepine and 4-DAMP (Research

Biochemicals, Inc., Natick, MA).

Concentration-response curves of carbachol after a 50-min treatment with PrBCM (3×10^{-6} M) and those of pilocarpine without PrBCM-treatment were shifted to the right in a parallel manner by the four antagonists, pirenzepine, AF-DX 116, himbacine and 4-DAMP. The pA₂ values and slopes of the four antagonists are shown in Table 1. Schild plots of these results yielded a straight line with a slope of 1 (Table 1). The pA₂ values of the four antagonists against pilocarpine were not different from their values against carbachol after the treatment with PrBCM (Table 1). The results suggest that the PrBCM-sensitive muscarinic cholinceptors are of the PrBCM-resistant subtype.

It is widely known that pirenzepine has its highest affinity for m1 (M₁) receptors; AF-DX 116, for m2 (M₂) receptors; and 4-DAMP, for m1 (M₁) and m3 (M₃) receptors (14). Our results suggest that the subtype of the PrBCM-sensitive and -resistant muscarinic cholinceptors is not the m1 (M₁) or m2 (M₂) subtype, because pirenzepine and AF-DX 116 have low affinity (Table 1). Dörje et al. (10) reported that himbacine is a valuable tool for distinguishing m2 and m4 receptors from all other subtypes, m1, m3 and m5. The pA₂ values of himbacine against carbachol after the treatment with PrBCM and pilocarpine are 7.00 ± 0.02 and 7.02 ± 0.03 , respectively (Table 1). These values are in good agreement with the pK_i value (7.03 ± 0.03) for m3 receptors reported by Dörje et al. (10). However, these values are significantly different from the pK_i values (7.96 ± 0.05 and 6.31 ± 0.05) for m4 and m5 receptors, respectively, reported by Dörje et al. (10). Moreover, the pA₂ values of 4-DAMP support that the subtype of PrBCM-sensitive and PrBCM-resistant muscarinic cholinceptors is the m3 subtype. These results suggest that the subtype of PrBCM-sensitive and -resistant muscarinic cholinceptors in guinea pig ileal muscle is the m3 subtype and not the other subtypes, m1, m2, m4 and m5. Recently, we reported that PrBCM

Table 1. The pA₂ values and slopes for four muscarinic antagonists

Antagonists	PrBCM-sensitive receptors (against pilocarpine)		PrBCM-resistant receptors (against carbachol after treatment with PrBCM)	
	pA ₂	slope	pA ₂	slope
Pirenzepine	6.56 ± 0.04	1.00 ± 0.12	6.49 ± 0.02	0.94 ± 0.01
AF-DX 116	6.45 ± 0.04	1.03 ± 0.10	6.30 ± 0.06	0.90 ± 0.10
Himbacine	7.02 ± 0.03	0.97 ± 0.01	7.00 ± 0.02	0.96 ± 0.01
4-DAMP	9.05 ± 0.03	0.96 ± 0.01	9.00 ± 0.04	0.95 ± 0.02

Mean $\pm$ S.E. of 6 experiments.

differentiated the two mechanisms, PrBCM-sensitive and -resistant ones, in the presence of guanine nucleotide (GTP), but in its absence, PrBCM did not discern the two. In other words, one mechanism is PrBCM-sensitive, irrespective of the presence or absence of GTP, and the other is PrBCM-resistant only when GTP is present (15). G-Protein-linked receptors and G-protein are considered to be present separately, and the receptors are activated by their binding with an agonist; the receptors and G-protein will associate to elicit a final response. However, it is now not known how GTP regulates PrBCM-sensitive and -resistant cholinergic receptors. Since the subtype of muscarinic cholinergic receptors in guinea pig ileal muscle is only the m3 subtype, PrBCM may distinguish between two states of m3 with and without GTP, although further studies are needed to clarify this point.

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REFERENCES

- 1 Hammer, R., Berrie, P.C., Birdsall, N.L.M., Burgen, A.S.V. and Hulme, E.C.: Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* **283**, 90–92 (1980)
- 2 Hammer, R., Giraldo, E., Schiavi, G.B., Monferini, E. and Ladinsky, H.: Binding profile of a novel cardioselective muscarinic receptor antagonist, AF-DX 116, to membranes of peripheral tissues and brain in the rat. *Life Sci.* **38**, 1653–1662 (1986)
- 3 De Jonge, A., Doods, H.N., Riesbons, D. and Van Zwieten, P.A.: Heterogeneity of muscarinic binding sites in rat brain, submandibular gland and atrium. *Br. J. Pharmacol.* **89**, 551P (1986)
- 4 Doods, H.N., Mathy, M.J., Davidesko, D., Van Carldorp, K.L., De Jonge, A. and Van Zwieten, P.A.: Selectivity of muscarinic antagonists in radioligand and in vivo experiments for putative M₁, M₂ and M₃ receptors. *J. Pharmacol. Exp. Ther.* **242**, 257–262 (1987)
- 5 Takayanagi, I., Kiuchi, Y., Ohtsuki, H. and Harada, M.: Activation of propylbenzilylcholine mustard-sensitive muscarinic cholinergic receptors more effectively utilizes cytosolic Ca²⁺ for contraction in guinea-pig intestinal smooth muscle. *Eur. J. Pharmacol.* **187**, 139–142 (1990)
- 6 Takayanagi, I., Harada, M. and Koike, K.: A difference in receptor mechanisms for muscarinic full and partial agonists. *Japan. J. Pharmacol.* **56**, 23–31 (1991)
- 7 Takayanagi, I., Ohtsuki, H., Saito, K., Koike, K. and Satoh, M.: Propylbenzilylcholine mustard (PrBCM)-sensitive cholinergic receptors and contractile response to partial agonist in guinea pig ileal muscle. *Japan. J. Pharmacol.* **56**, 151–158 (1991)
- 8 Bonner, T.I., Buckley, N.J., Young, A.C. and Brann, M.R.: Identification of a family of muscarinic acetylcholine receptor genes. *Science* **237**, 527–532 (1987)
- 9 Fukuda, K., Kubo, T., Maeda, A., Akiba, I., Bujo, H., Nakai, J., Mishina, M., Higashida, H., Neher, E., Marty, A. and Numa, S.: Selective effector coupling of muscarinic acetylcholine receptor subtypes. *Trends Pharmacol. Sci.* **10**, Supp. 4–10 (1989)
- 10 Dörje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E. and Brann, M.R.: Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J. Pharmacol. Exp. Ther.* **256**, 727–733 (1991)
- 11 Hisayama, T., Kumagai, N. and Takayanagi, I.: Two apparently distinct muscarinic cholinergic mechanisms in guinea-pig taenia caecum. *Japan. J. Pharmacol.* **46**, 414–416 (1988)
- 12 Arunlakshana, O. and Schild, H.O.: Some quantitative uses of drug antagonist. *Br. J. Pharmacol.* **14**, 48–58 (1959)
- 13 Tallarida, R.J., Cowan, A. and Adler, M.W.: pA₂ and receptor differentiation: a statistical analysis of competitive antagonism. *Life Sci.* **25**, 637–654 (1979)
- 14 Hulme, E.C., Birdsall, N.J.M. and Buckley, N.J.: Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **30**, 633–673 (1990)
- 15 Kiuchi, Y., Kumagai, N., Hisayama, T. and Takayanagi, I.: Guanosine 5'-triphosphate converts some populations of propylbenzilylcholine mustard-sensitive muscarinic cholinergic sites to sites resistant to the drug in intestinal smooth muscle. *Japan. J. Pharmacol.* **55**, 329–338 (1991)