Pharmacological Profiles of Muscarinic Receptors in the Longitudinal Smooth Muscle of Guinea Pig Ileum

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ABSTRACT—We have examined the pharmacological subtypes of muscarinic receptors mediating phosphoinositide hydrolysis and contraction in the longitudinal smooth muscle of guinea pig ileum with the use of muscarinic antagonists. Carbachol increased the formation of 3H-inositol phosphates (IPs) in a dose-dependent manner in both ileal smooth muscle and the frontal cortex of rats. The rank order of muscarinic antagonists for inhibition of IP formation induced by carbachol was 4-DAMP = atropine > pirenzepine > AF-DX 116 in guinea pig ileal smooth muscle. In ileal smooth muscle, the inhibition by the M3 antagonist pirenzepine was about 15 times less than that by atropine. However, in the rat frontal cortex, the inhibition by pirenzepine was only about 3 times less than that by atropine. The inhibitory effect of the M2 antagonist AF-DX 116 was weak in both ileal muscle and the frontal cortex. The M3 antagonist 4-DAMP strongly inhibited carbachol-induced IP formation in ileal smooth muscle. The rank order of muscarinic antagonists for inhibition of contraction induced by 10^-7 M carbachol was atropine >= 4-DAMP > pirenzepine > AF-DX 116. These results suggest that both IP formation and the contractile response induced by muscarinic agonists are mediated through the muscarinic M3 subtype in guinea pig ileum.

Keywords: Ileum (guinea pig), Muscarinic antagonist, Phosphoinositide response, Muscarinic M3 subtype
**Tissue preparation**

Guinea pigs were decapitated, and the small intestine was promptly removed and placed in oxygenated Krebs-Ringer bicarbonate buffer solution (KRB) of the following composition: 123 mM NaCl, 5.0 mM KCl, 1.3 mM MgCl₂, 1.4 mM KH₂PO₄, 26.0 mM NaHCO₃, 0.8 mM CaCl₂ and 1.0 mM glucose. The longitudinal muscle layer was then isolated.

Rats were killed by decapitation, and their brains were rapidly removed. Thin sections (400-μm thickness) were cut with a McIlwain tissue chopper in a cold room. Samples of the frontal cortex were obtained with a micro-puncture technique (20).

**Formation of ³H-inositol phosphates (IPs)**

Experiments on ³H-IP formation were performed as described previously (21). The longitudinal muscle layer was cut into 20 pieces of approximately 10-mm length, which were preincubated for 10 min in KRB at 37°C under 5% CO₂–95% O₂ and then prelabelled with 30 μCi of d-myo-³H-inositol in 5 ml of KRB for 3 hr at 37°C. The labelled strips were washed with KRB to remove excess d-myo-³H-inositol and then weighed. The frontal cortex of rats was also prelabelled with 3 μCi of d-myo-³H-inositol at 37°C for 90 min.

Prelabelled longitudinal muscle and rat frontal cortex were preincubated for 10 min in KRB solution in which NaCl was replaced by 10 mM LiCl. Muscarinic agonists were added to the KRB solution containing LiCl and incubated under 5% CO₂–95% O₂ at 37°C for 30 min (longitudinal muscle) or 60 min (frontal cortex). The muscarinic antagonists were applied before incubation with the agonists. Reactions were terminated by addition of ice-cold chloroform/methanol (1:2 by v/v). The mixtures were then homogenized in a glass homogenizer (longitudinal muscle) or with a sonicator (frontal cortex). The homogenates were mixed with equal volumes of water and chloroform and then centrifuged at 1,000 × g for 10 min. The upper aqueous phase was removed for measurement of ³H-IPs. ³H-IPs were separated by anion exchange chromatography by the method of Berridge et al. (22). ³H-IPs were separated on Dowex AG-X 8 resin (formate form), and aliquots were counted by liquid scintillation spectrometry (LKB: Pharmacia Biotech., Uppsala, Sweden). Values of IPs are expressed as percent increases over the basal level.

ED₅₀ and IC₅₀ values were determined by Hill plots. Protein in the frontal cortex was measured by the method of Lowry et al. (23).

**Contractile response**

The ileum was removed, and the longitudinal smooth muscle was carefully stripped off. Smooth muscle strips of 1.5-cm length were mounted in an organ bath containing 5 ml of KRB (127 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃ and 10.0 mM glucose), which was gassed with a mixture of 95% O₂ and 5% CO₂. The buffer was maintained at 37°C, and a mixture of 95% O₂ and 5% CO₂ was bubbled through it continuously. The tissue was maintained under a resting tension of 1 g. Contractile responses to drugs were recorded with a transducer (San-eci, Tokyo), and mean values were calculated as percentages of the maximum response evoked by 10⁻⁷ M carbachol.

**Drugs**

d-myo-³H-Inositol (specific activity, 703 GBq/mmol) was obtained from Amersham (Buckinghamshire, UK). Atropine and carbachol were purchased from Merck (Darmstadt, Germany). Oroxtenorine sesquifumarate was from Sigma (St. Louis, MO, USA). 4-DAMP was from Research Biochemicals (Natick, MA, USA). Pirenzepine and AF-DX 116 were gifts from Boehringer Ingelheim (Ingelheim am Rhein, Germany).

**RESULTS**

In a preliminary experiment, we examined the time courses of the formations of inositol monophosphate (IP₁), inositol bisphosphate (IP₂) and inositol trisphosphate (IP₃) induced by carbachol (10⁻⁴ M) in guinea pig ileal smooth muscle. Carbachol induced linear increases of SH IP₁, IP₂ and IP₃ formation for 30 min after its addition in smooth muscle and for 60 min in rat frontal cortex (data not shown). In this study, PI hydrolysis was expressed as the total amount of ³H-IPs (IP₁, IP₂ and IP₃), since IP₁ constituted more than 80% of the total inositol phosphates on stimulation with muscarinic agonists in both tissues.

The relative potencies of muscarinic agonists to stimulate formations of IPs in guinea pig ileal smooth muscle and rat frontal cortex were compared (Fig. 1: A, B and Table 1). Dose-dependent increase in the formation of ³H-IPs were observed with carbachol in both the ileal smooth muscle and the frontal cortex. However, oxotremorine was a very weak stimulator of IP formation in the rat frontal cortex (Fig. 1B).

Muscarinic antagonists dose-dependently inhibited the carbachol-induced formation of IPs. As shown in Fig. 2, the rank order of the IC₅₀ values of muscarinic antagonists for IP formation was 4-DAMP = atropine > pirenzepine > AF-DX 116 in the ileal smooth muscle of guinea pigs. In the ileal muscle, the inhibition by the M₁ antagonist pirenzepine was 14.8-fold less than that by atropine, whereas, in the rat frontal cortex, the inhibition...
by pirenzepine was only 2.8-fold less than that by atropine (Fig. 2 and Table 2). Thus pirenzepine was more effective in the frontal cortex (IC₅₀: 8.44 × 10⁻⁸ M) than in ileal smooth muscle (IC₅₀: 3.77 × 10⁻⁷ M). The inhibitory abilities of the M₂ antagonist AF-DX 116 were weak in both the ileal smooth muscle (IC₅₀: 2.46 × 10⁻⁶ M) and frontal cortex (IC₅₀: 2.74 × 10⁻⁶ M). The M₃ antagonist 4-DAMP strongly inhibited carbachol-induced IP formation in ileal smooth muscle (IC₅₀: 1.52 × 10⁻⁸ M), showing almost the same potency as atropine. The rank order of muscarinic antagonists for inhibition of contraction induced by 10⁻⁷ M carbachol was atropine ≥ 4-DAMP > pirenzepine > AF-DX 116. This rank order of potency was similar to that for the inhibition of IP hydrolysis (Fig. 3 and Table 2).

### Table 1. EC₅₀ values of muscarinic agonists on formation of ³H-IPs

| Agonists       | Ileal muscle EC₅₀ (M) | Frontal cortex EC₅₀ (M) |
|----------------|-----------------------|-------------------------|
| Carbachol      | 2.82 × 10⁻⁵           | 4.93 × 10⁻⁴             |
| Oxotremorine   | 3.98 × 10⁻⁵           | 2.19 × 10⁻⁵             |

Mean EC₅₀ values were calculated from 3–5 separate determinations of the dose-response curves.

### Table 2. IC₅₀ values for muscarinic antagonists on carbachol-induced ³H-IP formation and contraction

| Antagonist  | IP formation | Contraction |
|-------------|--------------|-------------|
|             | Ileal muscle | Frontal cortex | Ileal muscle |
| Atropine    | 2.55 × 10⁻⁸  | 4.93 × 10⁻⁸  | 9.33 × 10⁻⁹  |
| 4-DAMP      | 1.52 × 10⁻⁸  | —            | 3.98 × 10⁻⁸  |
| Pirenzepine | 3.77 × 10⁻⁷  | 8.44 × 10⁻⁸  | 1.17 × 10⁻⁶  |
| AF-DX 116   | 2.46 × 10⁻⁶  | 2.74 × 10⁻⁶  | 3.55 × 10⁻⁶  |

Mean IC₅₀ values were calculated from 3–5 separate determinations of Hill plots.
DISCUSSION

In this study, we found that carbachol acts as a full agonist on the formation of IPs, whereas oxotremorine appears to be a week partial agonist in both guinea pig ileal smooth and the frontal cortex of rat. Similar findings on the effects of muscarinic agonists on IP formations have been obtained in the cerebral cortex (10, 24, 25), astrocytoma 1321 N1 cells (26), chick embryo heart (27), and guinea pig bladder and colon (28).

In the present study, we used the selective muscarinic antagonists pirenzepine, AF-DX 116 and 4-DAMP to examine the subtype of muscarinic receptors that mediates IP formation. The inhibitory effect of the M2 antagonist AF-DX 116 on carbachol-induced IP formation was weak in both the smooth muscle of guinea pig ileum and the frontal cortex of rats, suggesting that involvement of the cardiac M2 subtype is negligible in these tissues. The inhibitory effects of the M1 antagonist pirenzepine on carbachol-induced IP formations were greater in the frontal cortex than in the ileal smooth muscle of guinea pigs. Pirenzepine also has weaker effects on other smooth muscles than on brain tissues (27–31). Binding studies have also indicated that pirenzepine has very high affinity to the muscarinic receptor in the cerebral cortex of rat brain (2, 32). These observations suggest that the M1 subtype coupled to IP formation is predominant in the brain.

4-DAMP, which is thought to be a very selective antagonist of the M3 subtype (6, 7), strongly inhibited carbachol-induced IP formation in ileal smooth muscle, being more potent than pirenzepine and AF-DX 116 (Fig. 2).

Konno and Takayanagi have found that the muscarinic receptor linked to PI turnover is associated with the smooth muscle M2 subtype by experiments using a muscarinic antagonist, himbacine (18). It has been reported that the smooth muscle M2 subtype (M2Δ) corresponds to the M3 subtype (7, 33). However, the difference between the inhibitory potency of himbacine and that of the M1 antagonist pirenzepine on IP formations induced by carbachol is small. In this study, the M3 antagonist 4-DAMP strongly inhibited carbachol-induced IP formation in ileal smooth muscles, showing almost the same potency as atropine. Therefore, we can suggest that the muscarinic receptor subtype linked to PI turnover in guinea pig ileum is related to the M3 subtype. The inhibitory effect of 4-DAMP on the carbachol-induced contractile response was also the most potent (Fig. 3). These results suggest that IP formation in the ileum may be mediated through the M3 subtype, although the population of the M3 subtype is thought to be small in the tissue (15, 16, 34, 35). Further studies are needed to clarify the subtype of muscarinic receptors in the ileum using other specific agonists or antagonists, as functional M2 (m2) and m4 subtypes have recently been identified in the ileum by binding and immunological studies (15–17).

We conclude that IP formation and the contractile response seem to be mediated by the M3 subtype in guinea pig ileum, whereas IP formations in the rat frontal cortex are mediated by the M1 subtype.

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