The Mechanism of Muscarinic Agonist-Stimulated Inositol Phosphate Formation in Permeabilized Ileal Smooth Muscle

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ABSTRACT—Muscarinic agonists and guanylyl-5'-imidodiphosphate (Gpp(NH)p) stimulated formation of inositol phosphates in permeabilized longitudinal smooth muscle of guinea pig ileum. Gpp(NH)p markedly potentiated the formation of inositol bisphosphate (IP₂) and inositol trisphosphate (IP₃) stimulated by carbachol, but increased inositol monophosphate formation (IP₁) only slightly. Gpp(NH)p enhanced the formation of IP₂ + IP₃ induced by either acetylcholine or carbachol about fourfold in a synergistic manner, but enhanced the effects of oxotremorine and pilocarpine less than twofold in an additive manner. Elevation of Ca²⁺ concentration resulted in increases of the inositol phosphate levels stimulated by both carbachol and Gpp(NH)p. The optimal concentration of Ca²⁺ for carbachol-stimulated formations of IP₂ + IP₃ was shifted to a lower Ca²⁺ concentration in the presence of Gpp(NH)p. These findings suggest that muscarinic receptor-stimulated polyphosphoinositide hydrolysis in ileal smooth muscle results in inositol polyphosphate formation via GTP binding protein (G-protein). The muscarinic receptor-activated G-protein decreases the Ca²⁺ requirement of polyphosphoinositide hydrolysis. Muscarinic agonists stimulate inositol polyphosphate formation by interaction of the G-protein activation of a phosphoinositide specific phospholipase C with Ca²⁺ influx.

Keywords: Muscarinic agonist, Phosphoinositide response, Full agonist, G-protein, Ileum

Activation of the muscarinic receptor (mACh R) causes a variety of cellular responses, including inhibition of adenylate cyclase, activation of phospholipase C (PLC) and activation of K⁺ ion channels (1–4). The mACh R-mediated responses are known to occur by the interaction of receptors with GTP binding proteins (G-protein) in the membranes (5–9). Muscarinic agonists have been classified into two types, designated as full agonists and partial agonists, based on differences in the efficacy of inositol phosphate formation and adenylate cyclase inhibition. Fisher (10) suggested that the difference in the efficacies of agonists arises from conformational restraints imposed by the coupling state of the receptor and agonist. Several studies have demonstrated that various types of muscarinic agonists stimulate intracellular second messengers that cause contractile responses in longitudinal smooth muscle of guinea pig ileum (11, 12). We have reported that the M3 subtype of mACh R is associated with phosphoinositide hydrolysis in guinea pig ileum (13). In the smooth muscle of guinea pig ileum, however, it is unclear how the interaction of muscarinic agonist-receptor results in the difference of efficacy in inositol phos-

phate formation.

In the present study, we examined inositol phosphate formation mediated by different types of muscarinic agonists in the presence of unhydrolyzable GTP analogs, Gpp(NH)p, using permeabilized ileal smooth muscle, and we discussed the difference in the abilities of muscarinic agonists.

MATERIALS AND METHODS

Materials

Myo-(2-)³H-inositol (specific activity, 703 GBq/mmol) was obtained from Amersham (Buckinghamshire, UK). Pertussis toxin (PTX) and cholera toxin (CTX) were gifts from the Chemo-Sero-Therapeutic Research Institute (Kumamoto). The following reagents were obtained from the indicated commercial sources: ethylene glycol bis (β-amino-ethylether)-N,N,N',N'-tetra acetic acid (EGTA; Dojindo Laboratories, Kumamoto); pilocarpine hydrochloride (Wako Pure Chemical Industries, Osaka); guanylyl-5'-yl-imidodiphosphate (Gpp(NH)p) (Boehringer Mannheim, Mannheim, Germany); digitonin and carbachol

(Merck, Darmstadt, Germany); oxotremorine sesquifumarate and eserine (Sigma, St. Louis, MO, USA); acetylcholine chloride (Nacalai Tesque, Kyoto); and Dowex-1 (AG-1, 100-200 mesh, $\times 8$ in the formate form; Bio-Rad, Hercules, CA, USA).

Labelling with ^3H -inositol in ileal smooth muscle

Male Hartley guinea pigs (300–450 g), obtained from Kyudo (Kumamoto), were housed in a room at 20–25°C with a 12-hr light-dark cycle (light on from 7:00 a.m.). Guinea pigs were decapitated, and their ileum was promptly removed and placed in oxygenated Krebs-Ringer bicarbonate buffer (KRB) of the following composition: 123 mM NaCl, 5.0 mM KCl, 1.3 mM MgCl_2 , 1.4 mM KH_2PO_4 , 26.0 mM NaHCO_3 , 0.8 mM CaCl_2 and 10.0 mM glucose, pH 7.4.

Experiments on ^3H -inositol phosphate formation were performed as described previously (13). Briefly, 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% CO_2 –95% O_2 . Then the pieces were prelabeled with 10 μCi (370 kBq)/ml of myo- ^3H -inositol for 3 hr at 37°C. The labelled strips were washed with KRB to remove excess myo- ^3H -inositol and then weighed.

Formation of ^3H -inositol phosphates in permeabilized longitudinal muscles.

Longitudinal muscles, ^3H -labelled muscles, were preincubated in KRB for 10 min at 37°C and then permeabilized with the non-ionic detergent digitonin (20 μM) for 10 min at 37°C in Ca^{2+} -free Li-KRB, pH 7.4 containing 10 mM EGTA, in which NaCl was replaced by 10 mM LiCl (Li-KRB). After the permeabilized strips were washed three times in Ca^{2+} -free Li-KRB, they were transferred to plastic tubes and incubated with muscarinic agonists and Gpp(NH)p in 500 μl of oxygenated Li-KRB for 10 min at 37°C. Reactions were terminated by adding of 2 ml of ice-cold chloroform/methanol (1 : 2 by v/v).

The reaction mixtures were then homogenized in a glass homogenizer, and the homogenates were mixed with equal volumes of water and chloroform and centrifuged at $1,000 \times g$ for 10 min. The upper aqueous phase was applied to a AG1-X8 column (100–200 mesh, formate form, 50% w/v slurry), and ^3H -inositol phosphates were separated into ^3H -inositol monophosphate (^3H -IP₁), ^3H -inositol bisphosphate (^3H -IP₂) and ^3H -inositol trisphosphate (^3H -IP₃) by a stepwise gradient elution with ammonium formate/formic acid buffers as described by Berridge et al. (14). The radioactivity content of each fraction was counted in a liquid scintillation spectrometer (LKB1217, RACK β ETA).

Cell damage induced by 20 μM digitonin treatment was

examined by electron-microscopy. Ileal smooth muscle appeared to be morphologically intact after treatment with digitonin (data not shown).

Fig. 1. Time courses of carbachol (100 μM)- and Gpp(NH)p (300 μM)-stimulated inositol phosphate formation in permeabilized ileal smooth muscle. Increases of IP₁ (A), IP₂ (B) and IP₃ (C) are expressed as percentages of basal levels. The basal radioactivities (dpm/mg wet weight) recovered in inositol phosphates were 141 ± 23.3 dpm (IP₁), 25.9 ± 1.7 dpm (IP₂) and 18.7 ± 2.7 dpm (IP₃) after 10 min. Values are means for 3 to 4 experiments. ●: carbachol + Gpp(NH)p, ○: carbachol, ■: Gpp(NH)p.

RESULTS

Time courses for IP₁, IP₂ and IP₃ formation in permeabilized ileal muscles

Figure 1 shows the time course for formations of IP₁, IP₂ and IP₃ stimulated by carbachol (100 μ M) and Gpp(NH)p (300 μ M). The muscarinic agonist carbachol stimulated rapid formations of IP₁, IP₂ and IP₃ within 60 sec, and their levels persisted for at least 10 min. The carbachol-stimulated IP₂ formation in the first 60 sec was markedly enhanced in the presence of Gpp(NH)p.

Fig. 2. Combined effects of carbachol (100 μ M) and Gpp(NH)p (300 μ M) on formations of inositol phosphates in permeabilized ileal smooth muscle. Values are expressed as radioactivities (dpm/mg wet weight) and are means $\pm$ S.E. for 4 to 6 experiments.

Effects of Gpp(NH)p on muscarinic agonists-stimulated formation of inositol phosphates

Ileal smooth muscles were treated with the non-ionic detergent digitonin to make them permeable to GTP analogues. In permeabilized ileal smooth muscle, the stable analogue of GTP, Gpp(NH)p (300 μ M), markedly potentiated the formations of IP₂ and IP₃ induced by carbachol (100 μ M), but had little effect on IP₁ formation stimulated by carbachol (Figs. 1 and 2). IP₂ is formed from IP₃ and is also formed from PIP by activation of PLC. Therefore, there is no single substrate for IP₂ formation in this tissue; and so in this study, we determined the formation of IP₂ + IP₃ as inositol polyphosphate formation. Gpp(NH)p potentiated the formation of IP₂ + IP₃ dose-dependently (Fig. 3).

Next, we examined the effect of Gpp(NH)p on the formation of IP₂ + IP₃ stimulated by various muscarinic

Fig. 3. Concentration-response relationship of the potentiation of 100 μ M carbachol-stimulated formations of inositol phosphates by Gpp(NH)p in permeabilized ileal smooth muscle. The responses were measured in combination with increasing concentrations of Gpp(NH)p. Values are expressed as radioactivities (dpm/mg wet weight) and are means $\pm$ S.E. for 4 to 6 experiments. $\circ$: control, $\bullet$: carbachol.

(A) IP₁ (B) IP₂ + IP₃

Fig. 4. Comparison of potentiations by Gpp(NH)p (300 μ M) of the stimulation of inositol phosphate formation by various muscarinic agonists (100 μ M) in permeabilized ileal smooth muscle. Acetylcholine was tested with 1 μ M eserine to minimize its hydrolysis. Values are expressed as radioactivities (dpm/mg wet weight) and are means $\pm$ S.E. for 4 to 12 experiments. CCh: carbachol, ACh: acetylcholine, Oxot: oxotremorine, Pilo: pilocarpine. $\square$: control, $\boxtimes$: Gpp(NH)p.

(A) IP₁ (B) IP₂ + IP₃

Fig. 5. Effects of Ca²⁺ on formations of inositol phosphates in permeabilized ileal smooth muscle. Values are expressed as percentages of the maximal response of IP₁ (A) or IP₂ + IP₃ (B) stimulated by a combination of carbachol (100 μ M) and Gpp(NH)p (300 μ M) and are means for 3 to 5 experiments. $\square$: basal, $\blacksquare$: Gpp(NH)p, $\circ$: carbachol, $\bullet$: carbachol + Gpp(NH)p.

agonists. Gpp(NH)p enhanced the formation of IP₂ + IP₃ induced by either acetylcholine or carbachol about fourfold in a synergistic manner, but enhanced the effects of oxotremorine and pilocarpine less than twofold in an additive manner (Fig. 4).

Effects of Ca²⁺ on formation of inositol phosphates in permeabilized ileal smooth muscle

The requirement of Ca²⁺ for formation of inositol phosphates in permeabilized ileal smooth muscle is shown in Fig. 5. Elevation of Ca²⁺ resulted in increases in the level of inositol phosphates stimulated by carbachol and Gpp(NH)p. The carbachol-stimulated formation of IP₂ + IP₃ was greatly potentiated by Gpp(NH)p in the presence of 0.09 mM Ca²⁺. Interestingly, the optimal concentration of Ca²⁺ (0.18 mM) for carbachol-stimulated formation of IP₂ + IP₃ was decreased to 0.09 mM in the presence of Gpp(NH)p. However, the optimal concentration of Ca²⁺ for IP₁ formation stimulated by carbachol was not affected by Gpp(NH)p.

Fig. 6. Effects of extracellular Ca²⁺ on formations of IPs (inositol phosphates) by muscarinic agonists (100 μ M) in intact ileal smooth muscle. ³H-Labelled ileal smooth muscles were incubated with muscarinic agonists for 10 min in Li-KRB under 5% CO₂ - 95% O₂ at 37°C. Values are expressed as radioactivities (dpm/mg wet weight) and are the means $\pm$ S.E. for 3 experiments. IPs: IP₁ + IP₂ + IP₃, CCh: carbachol, Oxot: oxotremorine, Pilo: pilocarpine. $\square$: Ca²⁺ free (0.5 mM EGTA), $\boxtimes$: 6.5 μ M Ca²⁺, $\blacksquare$: 1.6 mM Ca²⁺.

Effects of Ca^{2+} on formation of inositol phosphates in intact ileal smooth muscle

Next, we examined the effect of extracellular Ca^{2+} concentration on the muscarinic agonists-stimulated inositol phosphates (Fig. 6). Inositol phosphate formation stimulated by oxotremorine was reduced 68% by a lower Ca^{2+} concentration ($6.5 \mu\text{M}$) compared with that at 1.6 mM Ca^{2+} . However, the level of inositol phosphate formation stimulated by either carbachol or pilocarpine was not significantly different between the lower and higher Ca^{2+} concentrations. Chelating extracellular Ca^{2+} with 0.5 mM EGTA in the buffer decreased the inositol phosphates formation stimulated by muscarinic agonists.

DISCUSSION

Muscarinic agonists have been classified into two groups: full agonists, such as acetylcholine, carbachol and muscarine, and partial agonists, such as oxotremorine, pilocarpine, arecoline and bethanechol. Previously, we demonstrated that the maximum responses of inositol phosphate formation induced by carbachol and oxotremorine in the ileum were different from those in the brain (13). In addition, it is well known that the activation of polyphosphoinositide hydrolysis stimulated by muscarinic agonists was mediated by the G-protein (15). Takayanagi et al. (16) reported that the inhibitory effects of Gpp(NH)p on the affinity of the partial agonists to muscarinic receptors were correlated with their intrinsic activity in contraction. However, direct evidence for the interaction between mAChR and G-protein in polyphosphoinositide hydrolysis in the ileal smooth muscle has not been provided.

Therefore, the present study showed that G-protein is involved in the inositol polyphosphate formation in ileal smooth muscle. As seen in Fig. 4, in the presence of Gpp(NH)p, full agonists, such as acetylcholine and carbachol, potentiated the inositol polyphosphate formation in a synergic manner. On the other hand, partial agonists, such as oxotremorine and pilocarpine, stimulated the formation in an additive manner. These results indicate that full agonists stimulate inositol polyphosphate formation mediated by the G-protein in ileal smooth muscle. The same results were observed in membrane preparations from coronary artery (17), iris sphincter smooth muscle (18) and cardiac muscle (19). In contrast, partial agonists may stimulate partially phosphoinositide hydrolysis in a G-protein-independent manner. Takayanagi et al. (20–22) demonstrated that there are two types of propylbenzylcholine mustard (PrBCM) sensitive- and PrBCM-resistant sites of M3 mAChR in ileal smooth muscle. They suggested that the partial agonist pilocarpine caused the contractile response through PrBCM-sensitive sites,

and the PrBCM-sensitive sites were not coupled with GTP. These findings suggest that pilocarpine stimulates contraction through muscarinic receptors that are not coupled to G-protein.

In this study, IP_2 formation stimulated by carbachol was rapidly and synergistically potentiated by Gpp(NH)p in permeabilized ileal smooth muscle, although carbachol-stimulated IP_3 formation was not affected by Gpp(NH)p in the early stage (Fig. 1). It is possible that muscarinic agonist-stimulated G-protein activation induces the two breakdown systems in ileal smooth muscle, phosphatidylinositol monophosphate hydrolysis and phosphatidylinositol bisphosphate hydrolysis. However, further experiments are necessary to examine this possibility.

Several reports demonstrated that muscarinic receptor-stimulated inositol phosphates formation is Ca^{2+} -dependent in the ileal smooth muscle (23, 24). In the present study, full and partial agonists did not cause inositol phosphates formation in Ca^{2+} -free buffer containing EGTA (0.5 mM) in intact guinea pig ileum (Fig. 6). These findings suggest that muscarinic agonists may activate PLC through Ca^{2+} influx in the ileal smooth muscle. However, pilocarpine and carbachol could elicit inositol phosphate formation at lower Ca^{2+} concentration ($6.5 \mu\text{M}$) as well as at higher Ca^{2+} concentration (1.6 mM). These results suggest the possibility that muscarinic agonists caused phosphoinositide hydrolysis through not only Ca^{2+} influx but also G-protein activation.

In permeabilized preparations, the formation of inositol phosphates stimulated by carbachol and Gpp(NH)p was also Ca^{2+} -dependent (Fig. 5). The carbachol-induced formation of inositol polyphosphate was potentiated by Gpp(NH)p when the Ca^{2+} concentration was raised from 0 mM to 0.09 mM Ca^{2+} . This finding suggests that activation of G-protein may increase the Ca^{2+} sensitivity of PLC activation. Polyphosphoinositide hydrolysis can be elicited even at a very low Ca^{2+} influx if it is accompanied by G-protein activation.

Several studies have indicated that the activation of PLC is coupled with either PTX-sensitive or -insensitive G-protein (25). In general, inositol phosphate formation stimulated by agonists at the muscarinic receptor seems to be coupled with a PTX-insensitive G-protein (17, 18, 26). Pretreatment of ileal smooth muscle with PTX and CTX did not affect carbachol-stimulated inositol polyphosphate formation (K. Honda et al., unpublished data).

In conclusion, the present study demonstrated that muscarinic receptor-stimulated polyphosphoinositide hydrolysis in ileal smooth muscle results in inositol polyphosphate formation via G-protein. Moreover, muscarinic receptor-stimulated G-protein activation increases the sensitivity of polyphosphoinositide hydrolysis to Ca^{2+} in

ileal smooth muscle. Muscarinic agonists stimulate inositol polyphosphates formation by the interaction of the G-protein activation of a phosphoinositide specific PLC with Ca^{2+} influx.

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