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

Kenji Honda, Yukio Takano and Hiro-o Kamiya

Department of Pharmacology, Faculty of Pharmaceutical Sciences, Fukuoka University, Fukuoka 814-01, Japan

Received February 24, 1994  Accepted April 18, 1994

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 (IP$_2$) and inositol trisphosphate (IP$_3$) stimulated by carbachol, but increased inositol monophosphate formation (IP$_1$) only slightly. Gpp(NH)p enhanced the formation of IP$_2$ + IP$_3$ 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 Ca$^{2+}$ concentration resulted in increases of the inositol phosphate levels stimulated by both carbachol and Gpp(NH)p. The optimal concentration of Ca$^{2+}$ for carbachol-stimulated formations of IP$_2$ + IP$_3$ was shifted to a lower Ca$^{2+}$ 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 Ca$^{2+}$ requirement of polyphosphoinositide hydrolysis. Muscarinic agonists stimulate inositol polyphosphate formation by interaction of the G-protein activation of a phosphoinositide specific phospholipase C with Ca$^{2+}$ 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 phosphate 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-)$^3$H-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-ethyl ether)-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, × 8 in the formate form; Bio-Rad, Hercules, CA, USA).

Labelling with $^3$H-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$_2$PO$_4$, 26.0 mM NaHCO$_3$, 0.8 mM CaCl$_2$ and 10.0 mM glucose, pH 7.4.

Experiments on $^3$H-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-$^3$H-inositol for 3 hr at 37 °C. The labelled strips were washed with KRB to remove excess myo-$^3$H-inositol and then weighed.

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

Longitudinal muscles, $^3$H-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 × 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 $^3$H-inositol phosphates were separated into $^3$H-inositol monophosphate ($^3$H-IP$_1$), $^3$H-inositol bisphosphate ($^3$H-IP$_2$) and $^3$H-inositol trisphosphate ($^3$H-IP$_3$) 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$eta$ 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 pM)- and Gpp(NH)p (300 pM)-stimulated inositol phosphate formation in permeabilized ileal smooth muscle. Increases of IP$_1$ (A), IP$_2$ (B) and IP$_3$ (C) are expressed as percentages of basal levels. The basal radioactivities (dpm/mg wet weight) recovered in inositol phosphates were 141 ± 23.3 dpm (IP$_1$), 25.9 ± 1.7 dpm (IP$_2$) and 18.7 ± 2.7 dpm (IP$_3$) 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 μM) and Gpp(NH)p (300 μM). 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.

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 μM), markedly potentiated the formations of IP₂ and IP₃ induced by carbachol (100 μM), 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
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 oxtremorine and pilocarpine less than twofold in an additive manner (Fig. 4).

Effects of Ca\(^{2+}\) on formation of inositol phosphates in permeabilized ileal smooth muscle

The requirement of Ca\(^{2+}\) for formation of inositol phosphates in permeabilized ileal smooth muscle is shown in Fig. 5. Elevation of Ca\(^{2+}\) 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 Ca\(^{2+}\). Interestingly, the optimal concentration of Ca\(^{2+}\) (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 Ca\(^{2+}\) for IP1 formation stimulated by carbachol was not affected by Gpp(NH)p.

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

Fig. 5. Effects of Ca\(^{2+}\) 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 \(\mu\)M) and Gpp(NH)p (300 \(\mu\)M) and are means for 3 to 5 experiments. □: basal, ■: Gpp(NH)p, ○: carbachol, ●: carbachol + Gpp(NH)p.

Fig. 6. Effects of extracellular Ca\(^{2+}\) on formations of IPs (inositol phosphates) by muscarinic agonists (100 \(\mu\)M) in intact ileal smooth muscle. \(^{3}\)H-Labelled ileal smooth muscles were incubated with muscarinic agonists for 10 min in Li-KRB under 5% CO\(_2\) - 95% O\(_2\) at 37°C. Values are expressed as radioactivities (dpm/mg wet weight) and are the means ± S.E. for 3 experiments. IPs: IP1 + IP2 + IP3, CCh: carbachol, Oxot: oxtremorine, Pilo: pilocarpine. □: Ca\(^{2+}\) free (0.5 mM EGTA), ■: 6.5 \(\mu\)M Ca\(^{2+}\), ■: 1.6 mM Ca\(^{2+}\).
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 \(\mu\)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 mACh R 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 propylbenzil-ylcholine mustard (PrBCM) sensitive- and PrBCM-resistant sites of M3 mACh R 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 \(\mu\)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.

Acknowledgments
This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan. We thank Mr. Y. Nakayama, Ms. Y. Nakano, Ms. A. Nakano, Mr. K. Kondo, Mr. M. Ohtuka, Mr. M. Shibata and Mr. T. Makihira for technical assistance. We are grateful to the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan) for their generous gifts of pertussis toxin and cholera toxin.

REFERENCES

1 Nathanson NM: Molecular properties of the muscarinic acetylcholine receptor. Annu Rev Neurosci 10, 195–236 (1987)
2 Peralta EF, Ashkenazi A, Winslow JW, Ramachandra J and Capon DJ: Differential regulation of PI hydrolysis and adenylate cyclase by muscarinic receptor subtypes. Nature 334, 434–437 (1988)
3 Fukuda K, Kubo T, Akiba I, Maeda A, Mishina M and Numa S: Molecular distinction between muscarinic acetylcholine receptor subtypes. Nature 327, 623–625 (1987)
4 Gil DW and Wolfe BB: Pirenzepine distinguishes between muscarinic receptor-mediated phosphoinositide breakdown and inhibition of adenylate cyclase. J Pharmacol Exp Ther 232, 608–616 (1985)
5 Berrie CP, Birdsall EC, Hulme EC, Keen M and Stockton JM: Solubilization and characterization of guanine nucleotide-sensitive muscarinic agonist binding site from rat myocardium. Br J Pharmacol 82, 853–861 (1984)
6 Pfaffinger PJ, Martin JM, Hunter DD, Nathanson NM and Hille B: GTP-binding proteins couple cardiac muscarinic receptors to a K channel. Nature 317, 536–538 (1985)
7 Martin JM, Hunter DD and Nathanson NM: Islet activating protein inhibits physiological responses evoked by cardiac muscarinic acetylcholine receptors: role of guanosine triphosphate binding proteins in regulation of potassium permeability. Biochemistry 24, 7521–7525 (1985)
8 Martin JM, Subers SW, Halvorsen SW and Nathanson NM: Functional and physical properties of chick aortic and ventricular GTP-binding proteins. J Pharmacol Exp Ther 240, 683–688 (1987)
9 Moscona-Amir E, Henis HJ and Sokolovsky M: Guanosine 5'-triphosphate binding protein (G) and two additional pertussis toxin substrates associated with muscarinic receptors in rat heart myocytes: characterization and age dependency. Biochemistry 27, 4985–4991 (1988)
10 Fisher SK: Inositol lipids and signal transduction at CNS muscarinic receptors. Trends Pharmacol Sci 6, Supp 61–65 (1986)
11 Konno F and Takayanagi I: Relationship between the contractile responses and coupling second messenger systems for muscarinic drugs in the guinea pig ileal longitudinal muscle. Arch Int Pharmacodyn Ther 301, 15–19 (1989)
12 Wang XB, Osugi T and Uchida S: Different pathways for Ca$^{2+}$ influx and intracellular release Ca$^{2+}$ mediated by muscarinic receptors in ileal longitudinal smooth muscle. Jpn J Pharmacol 58, 407–415 (1992)
13 Honda K, Takano Y and Kamiya H: Pharmacological profiles of muscarinic receptors in the longitudinal smooth muscle of guinea pig. Jpn J Pharmacol 62, 43–47 (1993)
14 Berridge MJ, Dawson CP, Downes CP, Heslop JP and Irvine R: Change in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositide. Biochem J 212, 473–482 (1983)
15 Birnbaumer L, Abramowitz J and Brown M: Receptor-effector coupling by G proteins. Biochem Biophys Acta 1031, 163–224 (1990)
16 Takayanagi I, Koike K and Okumura K: Intrinsic activity and effects of guanyl-5'-yl imidodiphosphate, Gpp(NH)p on the affinity of partial agonists to the muscarinic receptor. Eur J Pharmacol 99, 107–110 (1984)
17 Sasaguri T, Hirata M, Itoh T, Koga T and Kuriyama H: Guanine nucleotide binding protein involved in muscarinic responses in the pig coronary artery is insensitive to islet-activating protein. Biochem J 239, 567–574 (1986)
18 Honkanen RE and Abdel-Latif AA: Muscarinic-agonist and guanine nucleotide stimulation of myo-inositol trisphosphate formation in membranes isolated from bovine iris sphincter smooth muscle: Effects of short-term cholinergic desensitization. Membr Biochem 8, 39–59 (1989)
19 Jones LG, Goldstein D and Brown JH: Guanine nucleotide-dependent inositol trisphosphate formation in chick heart cells. Circ Res 62, 299–305 (1988)
20 Harada M, Koike K and Takayanagi I: Characterization of subtype of propylbenzilcholine mustard (PrBCM)-sensitive and -resistant muscarinic cholinceptors in guinea pig ileal muscle. Jpn J Pharmacol 59, 485–487 (1992)
21 Takayanagi I, Harada M and Koike K: A difference in receptor mechanisms for muscarinic full and partial agonist. Jpn J Pharmacol 56, 23–31 (1991)
22 Kiuchi Y, Kunagai N, Hisayama T and Takayanagi I: Guanosine 5'-triphosphate converts some populations of propylbenzilcholine mustard-sensitive muscarinic cholinceptor sites to sites resistant to the drug in intestinal smooth muscle. Jpn J Pharmacol 55, 329–338 (1991)
23 Best L, Brooks KJ and Bolton TM: Relationship between stimulated inositol lipid hydrolysis and contractility in guinea pig visceral longitudinal smooth muscle. Biochem Pharmacol 34, 2297–2301 (1986)
24 Watson SP, Lai J and Sasaguri T: K$^+$-stimulation of the phosphoinositide pathway in guinea-pig ileum longitudinal smooth muscle is predominantly neuronal in origin and mediated by the entry of extracellular Ca$^{2+}$. Br J Pharmacol 99, 212–216 (1990)
25 Cockcroft S and Thomas GMH: Inositol-lipid-specific phospholipase C isoenzymes and their differential regulation by receptors. Biochem J 288, 1–14 (1992)
26 Master SB, Martin MW, Harden TK and Brown JH: Pertussis toxin does not inhibit muscarinic-receptor-mediated phosphoinositide hydrolysis or calcium mobilization. Biochem J 227, 933–937 (1985)