Possible Involvement of M₅ Muscarinic Receptor in the Enhancing Actions of the Novel Gastroprokinetic Agent Z-338 on Nifedipine-Sensitive Voltage-Dependent Ca²⁺ Currents in Guinea Pig Stomach

Hiromitsu Morita¹, Kihachiro Abe², Yushi Ito¹ and Ryuji Inoue¹,*

¹Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, Fukuoka 812-8582, Japan
²Special Patient Oral Care Unit of Kyushu University Dental Hospital, Fukuoka 812-8582, Japan

Received March 20, 2002 Accepted May 8, 2002

ABSTRACT—We investigated the effects of the novel gastroprokinetic agent Z-338 (N-(N'-N'-diisopropylaminoethyl)-[2-(2-hydroxy-4,5-dimethoxybenzoylamino)-1,3-thiazole-4-yl] carboxyamide monohydrochloride trihydrate) on L-type voltage-dependent Ca²⁺ currents (I₅ᵥ) in guinea pig gastric myocytes by using the whole-cell patch clamp technique. Bath-applied acetylcholine (ACh) produced biphasic effects on I₅ᵥ, i.e., enhancement (1 – 100 nM) and inhibition (1 – 100 μM), both of which were abolished by pretreatment with atropine (10 μM) or intracellular perfusion of GDP/βS (500 μM). Z-338 (≥1 nM, ED₅₀: 120 nM) mimicked the enhancing effects of ACh, but did not inhibit I₅ᵥ. The effects of Z-338 and ACh were non-additive and blocked by atropine and GDP/βS, but not by pertussis toxin (PTX) pretreatment (500 ng/ml). ACh (≥1 μM) induced slow inward currents via activation of the muscarinic receptor/PTX-sensitive G-protein pathway, but Z-338 was devoid of these effects. Neither pirenzepine (1 μM), AF-DX116 (1 μM), nor oxybutynin (100 nM) could prevent Z-338 (1 μM) and ACh (10 μM) from enhancing I₅ᵥ, whilst 4-DAMP (100 nM) blocked the effects of Z-338 and ACh. Bath-application of protein kinase C (PKC) activator PDBu (phorbol-12,13-dibutyrate) (250 nM) enhanced I₅ᵥ, and conversely, pipette inclusion of PKC inhibitor peptide (150 μM) abolished the effects of ACh and Z-338 on I₅ᵥ. These results collectively suggest that although contribution of the M₁ receptor is not excluded, the major actions of Z-338 on gastric myocytes are potentiation of I₅ᵥ through activation of M₅-like receptor.

Keywords: Z-338, M₅ muscarinic receptor, L-type calcium channel, Gastric smooth muscle

Z-338, a novel carboxyamide derivative, has recently been synthesized, in the hope to obtain more potent and clinically safer gastroprokinetic agents having a different mechanism from those of existing gastroprokinetic agents such as cisapride and domperidone, which have been reported to show unfavorable adverse effects on the central nervous and cardiovascular systems (1). It has been shown that Z-338 enhances gastric motor activity in conscious dogs with chronically implanted force transducers and restores gastric emptying suppressed by eloxidine treatment in both dog and rat gastroparesis models (1). The mechanisms involved in theses actions of Z-338 have been investigated in more detail by a number of different techniques. In guinea pig stomach strips preincubated with [³H]-labelled choline, Z-338 enhances the electrically stimulated release of [³H]-ACh. This is likely to occur through inhibition of M₁ and M₅ receptors, since Z-338 can bind to M₁ and M₅ (but not M₇) receptors prepared from rat tissues and effectively block membrane currents associated with activation of recombinantly expressed rat M₁ and M₅ receptors in Xenopus oocytes (2). Furthermore, it was also found that Z-338 enhances spontaneous contractions as well as electrically evoked excitatory junction potentials and concomitant contractions in guinea pig stomach with no significant changes in inhibitory neuro-
transmission, resting membrane potential or basal muscle tone (3). All these findings are consistent with the view that Z-338 facilitates the excitatory neurotransmission in the stomach by increasing ACh release from cholinergic nerve varicosities via inhibition of pre-junctional M₁ and M₂ receptors. However, it is still unclear what post-junctional mechanisms are involved in the observed increase in spontaneous motor activities of gastric smooth muscle upon application of Z-338. It is also puzzling why Z-388 is able to enhance spontaneous contractions without changing the basal tension level, while ACh affects both. Thus, in the present study, in order to clarify these points, we have investigated the ionic mechanisms underlying the excitatory actions of Z-338 via muscarinic receptors in comparison with those of ACh, by applying the patch clamp technique to single myocytes freshly dissociated from the stomach. Our results show that this compound potentiates the activity of the L-type Ca²⁺ channel almost exclusively through stimulation of a muscarinic receptor pharmacologically similar to the M₃ receptor.

MATERIALS AND METHODS

Cell dispersion

Hartley guinea pigs of either sex (400 to 700 g) were stunned and killed by decapitation. The whole stomach of each animal was excised out and cut open along the major curvature and divided into two parts. Each part was pinned stretched on the rubber bottom of a dissecting dish filled with 1 mM Ca²⁺ containing Krebs solution (for ionic composition see below). The gastric smooth muscle layer was cleaned of attached mucosa, vessels, and connective tissues, and minced into small pieces using fine scissors and forceps. These pieces were incubated in nominally Ca²⁺-free Krebs solution at 36°C for 30 min and transferred consecutively into the Ca²⁺ free Krebs solution supplemented with 2 mg/ml collagenase (type I; Sigma, St. Louis, MO, USA) at 36°C for 60 min. Single cells were dispersed by triturating the digested pieces with a large bored Pasteur pipette in Ca²⁺-free Krebs solution and stored in a refrigerator at 4 – 10°C until use. Experiments were carried out within 3 h from the time of cell dispersion. For PTX treatment, strips made from adjacent regions of the same gastric muscle were incubated in Krebs solution supplemented with or without 500 ng/ml PTX at 4 – 10°C for 12 – 18 h (the latter was taken as the control), from which single myocytes were dispersed following the protocol mentioned above. All procedures described above were performed according to the guidelines approved by a local animal ethics committee of Kyushu University.

Electrophysiology

The details of electrophysiological recordings in this study were essentially the same as described previously (4). Briefly, a low-noise, high-impedance patch clamp amplifier (Axopatch 1D; Axon Instruments, Union City, CA, USA) driven by an IBM-compatible computer (Aptiva) was used to generate voltage and acquire current signals, in conjunction with an A/D, D/A converter (TL-1, Axon Instruments). Obtained current signals were low-pass filtered (1 kHz), digitized (2 kHz), and stored on a computer hard disk. The leak subtraction procedure was routinely employed using the P/2 protocol where voltage pulses of half amplitude with an opposite polarity were applied prior to the test pulses. The series resistance of cells examined stayed almost constant throughout the experiments (5 – 7 MΩ), 50 – 70% of which was electronically compensated. Data were analyzed offline and an illustration made by the commercial software Clampfit version 6.04 (Axon Instruments) and KaleidaGraph version 3.04 (Synergy Software, Reading, PA, USA). In some cases in which long term recordings were needed, current signals were digitized every 10 ms using the MacLab (AD Instruments, Castel Hill, Australia). In some experiments such as the shown in Fig. 1C and 2B, the nystatin-perforated rather than conventional whole-cell recording was employed to examine the possible role of intracellular Ca²⁺ concentration. We found, however, little differences between the results obtained by the two modes of recording (Fig. 1C and Fig. 2B). We therefore employed the latter mode throughout the present study, in order to record I_Ca under better and more stable voltage-clamped conditions.

The temperature of the bathing solution was adjusted between 25 ± 1°C with an automatic temperature control unit (TC-344B; Warner Instruments, Hamden, CT, USA). The effects of agonists such as ACh, Z-338 and PDBu on I_Ca were evaluated after their equilibration, which normally took a few minutes, when I_Ca was repetitively evoked by 400-ms pulses at an interval of 20 s. When multiple concentrations were tested, cumulative application was employed. Inhibitors and antagonists were administered 10 min prior to the application of agonists. Considerable variations occurred in the magnitude of responses to ACh or Z-338, due probably to individual variations of experimental animals and a varying efficacy of enzymatic digestion. Particularly, overnight treatment of gastric muscle strips with or without PTX resulted in a significantly attenuated response of dissociated myocytes to ACh or Z-338 as well as a decrease in the current density of I_Ca, as can be seen in Fig. 4C. Thus, to minimize the influence of these variations, we compared the effects of given drugs between cells dissociated under identical enzymatic conditions from the same batches or adjacent regions of the same muscle strip (Figs. 3 – 5).
Solutions

The composition of solutions used in the present study are as follows: modified Krebs solution: 140 mM Na\(^+\), 6 mM K\(^+\), 1.2 mM Mg\(^2+\), 2 mM Ca\(^{2+}\), 151.4 mM Cl\(^-\), 10 mM glucose, 10 mM HEPES (adjusted to pH 7.4 with Tris base); Cs\(^+\) internal solution for whole-cell recording contained: 140 mM Cs\(^+\), 2 mM Mg\(^{2+}\), 144 mM Cl\(^-\), 5 mM phosphocreatine, 1 mM Na\(_2\)ATP, 10 mM EGTA and 10 mM HEPES (adjusted to pH 7.2 with Tris base); Cs\(^+\) internal solution for nystatin-perforated recording contained: 140 mM Cs\(^+\), 2 mM Mg\(^{2+}\), 144 mM Cl\(^-\), 1 mM EGTA, 10 mM glucose and 10 mM HEPES (adjusted to pH 7.2 with Tris base).

Chemicals

Z-338 was a kind gift from Zeria Pharmaceutical Co., Ltd. (Tokyo). ATP, GTP, HEPES, EGTA, TTX and nifedipine were purchased from Wako (Osaka); GDPβS, CdCl\(_2\) and oxybutynin, from Sigma; pirenzepine, AF-DX116 and 4-DAMP, from Nacalai Tesque (Kyoto); atropine and PTX, from Calbiochem (La Jolla, CA, USA).

Statistics

All data were expressed as the mean ± S.E.M. For statistical evaluation, two-tailed paired and unpaired t-tests or one way ANOVA with the pooled variance t-test were used where appropriate.

RESULTS

Complex actions of ACh on membrane currents

When voltage step pulses more positive than ~40 mV were applied to single gastric myocytes from a holding potential of ~60 mV with Cs\(^+\) (140 mM) in the pipette, rapidly activating and inactivating inward currents were elicited (Fig. 1: Aa, b; for I-V relationship, see Fig. 2C). These currents were not significantly affected by substitution of external Na\(^+\) by N-methyl-D-glucamine, but completely abolished in the presence of nifedipine (≥1 μM), suggesting that high voltage-activated L-type Ca\(^{2+}\) channels are responsible for its generation (I\(_{ca}\)). The properties of I\(_{ca}\) were in agreement with those previously reported in the same preparation (5, 6), but in some cells, transient inward currents showing much faster kinetics compared with I\(_{w}\) were recorded prior to the activation of I\(_{ca}\). These currents are likely to result from activation of voltage-dependent Na\(^+\) channels, since they were completely abolished in the presence of 1 μM TTX or upon total elimination of external Na\(^+\). Thus, in the rest of the present study, to avoid the contamination of voltage-dependent Na\(^+\) currents, 1 μM TTX was routinely added in the bath.

Figure 1A demonstrates typical actions of bath-applied ACh on the holding current and I\(_{ca}\) evoked by depolarizing pulses to 0 mV at two extreme concentrations. At a relatively low concentration of ACh (10 nM), there was almost no change in the basal current level, but the magnitude of I\(_{ca}\) was significantly enhanced. In contrast, at a high concentration (100 μM), a slow sustaining inward current developed after addition of ACh, and in parallel with this, the magnitude of I\(_{ca}\) decreased progressively (Fig. 1: Aa, b; for summary, see Fig. 1B). The reduction of I\(_{ca}\) at high ACh concentrations was due unlikely to superimposition of slow inward current, since its reversal potential was close to 0 mV (see the inset in Fig. 1C), at which I\(_{ca}\) was evaluated. Both enhancing and inhibiting effects of ACh occurred in a concentration-dependent fashion and did not seem to be strongly affected under weak Ca\(^{2+}\)-buffering conditions (open circles in Fig. 1C; data from nystatin-perforated recording). As summarized in Fig. 1C, the enhancing effects were already evident in the nanomolar range of ACh, whereas the effective concentrations of ACh to inhibit I\(_{ca}\) mostly spans the micromolar range (Fig. 1C). The magnitude of the slow sustaining inward current also depended on ACh concentration, the range of which almost coincided with that for I\(_{ca}\) inhibition (data not shown). The slow inward current was cationic (Na\(^+\) and Ca\(^{2+}\) removal abolished it) with a reversal potential of about 0 mV (inset in Fig. 1C), and its activation was strongly suppressed by pretreatment with AF-DX116 (1 μM), a relatively specific blocker for M\(_2\) muscarinic receptor subtype, or by overnight incubation of gastric muscle strips with PTX (500 ng/ml; see Materials and Methods) (data not shown). These results strongly suggest that the channels underlying the slow inward current are cation channels activated through stimulation of the M\(_2\) receptor/G\(_{ou}\) protein pathway that have ubiquitously been observed in the whole gastrointestinal tract (7, 8).

Z-338 solely enhances I\(_{ca}\)

Z-338 also enhanced I\(_{ca}\) in a concentration-dependent fashion (ED\(_{50}\) = 0.12 μM) (Fig. 2: A and B). However, different from ACh, Z-338 did not exhibit any discernible inhibition of I\(_{ca}\) even at a concentration as high as 100 μM (Fig. 2B), and it failed to evoke slow inward currents. The enhancing actions of Z-338 on I\(_{ca}\) are voltage-independent, as indicated by almost symmetrical current voltage relationships before and after application of ACh (Fig. 3C). Over a wide range of membrane potentials, the extent of I\(_{ca}\) potentiation by Z-338 remained nearly the same. Furthermore, the enhancing effects on I\(_{ca}\) (open circle in Fig. 2B) or inability to induce inward cationic currents of Z-338 were not changed, when the currents were recorded with nystatin-perforated recording, suggesting a less significant role of intracellular Ca\(^{2+}\) concentration in these effects. The effects of Z-338 and ACh to enhance I\(_{ca}\) appear to be mutually exclusive, since after I\(_{ca}\) had already been enhanced
M<sub>3</sub> Receptor Potentiates L-Type Ca<sup>2+</sup> Current

by 10 nM ACh, further application of Z-338 (1 μM) was ineffective, and vice versa (Fig. 3A). These results strongly suggest that common signaling pathways are involved in the enhancing actions of Z-338 and ACh.

The effects of ACh and Z-338 on I<sub>Ca</sub> are likely to occur through activation of muscarinic receptor/Go<sub>q/11</sub> G-protein /PKC pathways. Firstly, these effects were completely eliminated by bath application of atropine (10 μM) or intra-

---

**Fig. 1.** Biphasic actions of ACh on nifedipine-sensitive, L-type Ca<sup>2+</sup> current (I<sub>Ca</sub>) in guinea pig gastric myocytes. Bath and pipette contained a modified Krebs and Cs<sup>+</sup> internal solutions, respectively. A: Actual traces of holding current (b) and I<sub>Ca</sub> evoked by 400 ms depolarizing pulses (from −60 to 0 mV; a) in the absence and presence of 10 nM or 100 μM ACh. B: Averaged time course of the effects of ACh (10 nM and 100 μM) on I<sub>Ca</sub> from 5 separate experiments. C: Concentration-dependence of the effects of ACh on I<sub>Ca</sub> with conventional whole-cell (filled circles, n = 5) and nystatin-perforated (open circles, n = 6) recordings. Inset represents the averaged current-voltage relationship for ACh (100 μM)-induced current from 5 cells evaluated by 2 s rising ramp voltages in the presence of 10 μM nifedipine. In B and C, the amplitude of I<sub>Ca</sub> is normalized to that before application of ACh. *P<0.05, **P<0.01: difference from the control (dashed line), with paired t-test.
cellular perfusion of GDPβS (500 μM) via the pipette (Fig. 3B). Secondly, overnight incubation with PTX (500 ng/ml, see Materials and Methods) did not significantly affect the efficacy of ACh or Z-338 to cause potentiation and inhibition of $I_{Ca}$ (Fig. 4C), thus precluding an active role of $G_{i/o}$ G-protein in these signal transduction pathways. Thirdly, intracellular perfusion of PKC inhibitory peptide (150 μM) resulted in almost complete abolition of both enhancing and inhibitory actions of ACh and Z-338 on $I_{Ca}$ (Fig. 4A), and conversely, a potent PKC activator phorbol butyrate (PDBu, 250 nM) caused the potentiation of $I_{Ca}$, which occurred in a non-additive fashion to the effects of 1 μM Z-338 (Fig. 4B).

**Involvement of M₅ receptor in Z-338 induced $I_{Ca}$ potentiation**

To determine the muscarinic receptor subtypes involved in the enhancing and inhibitory actions of Z-338 and ACh, we examined the effects on $I_{Ca}$ of several widely used muscarinic antagonists having different subtype specificities (9, 10). These include pirenzepine (1 μM; relatively M₁ subtype-selective; pA₂ = 8.0), AF-DX116 (1 μM; rela-

---

**Fig. 2. Enhancing actions of Z-338 on $I_{Ca}$.** Recording conditions are the same as in Fig. 1. A: Actual trace of $I_{Ca}$ (a) and averaged time course of $I_{Ca}$ potentiation by 1 μM Z-338 (b, n = 5). B: Concentration-dependence of $I_{Ca}$ potentiation by Z-338 (filled circles). Solid curve indicates the result of Hill fitting of data points (n = 3–5). Open circle indicates the data obtained with the nystatin perforated recording (n = 5). C: Current-voltage relationships of $I_{Ca}$ before and during application and after washout of Z-338 1 μM. Representative of 4 separate experiments. In Ab and B, the amplitude of $I_{Ca}$ is normalized to that before application of Z-338.
M<sub>2</sub> Receptor Potentiates L-Type Ca<sup>2+</sup> Current

effectively M<sub>2</sub> subtype selective; pA<sub>2</sub> = 8.2 – 9.0), 4-DAMP (100 nM; almost equally effective for M<sub>1</sub>, M<sub>3</sub> and M<sub>5</sub> subtypes; pA<sub>2</sub> = 9.2, 9.2 and 8.9, respectively), and oxybutynin (100 nM; shows higher inhibitory efficacies for M<sub>1</sub>, M<sub>3</sub> and M<sub>4</sub> subtypes than for M<sub>2</sub> and M<sub>5</sub> subtypes; pA<sub>2</sub> = 8.5, 7.8, 8.7, 8.2 and 7.6 for M<sub>1</sub> – M<sub>5</sub> receptors, respectively). As summarized in Fig. 5, neither pirenzepine, AF-DX116, nor oxybutynin significantly altered, whereas 4-DAMP almost completely eliminated the enhancing effects of Z-338. Similarly, both potentiation (not shown) and inhibition (Fig. 5B) of I<sub>Ca</sub> by ACh were greatly reduced by pretreatment with 0.1 μM 4-DAMP (pirenzepine, AF-DX116; ineffective), but interestingly, in the presence of 0.1 μM oxybutynin, inhibition by high concentrations of ACh (100 μM) was reverted to potentiation, which was probably unmasked by elimination of concurrent inhibition. The extent of this potentiation did not become greater by further addition of Z-338 (Fig. 5B). However, when the concentration of oxybutynin was raised to greater than 1 μM, the potentiation was also significantly inhibited (data not shown). These results collectively indi-
cate that $I_{Ca}$ potentiation by Z-338 or low concentrations of ACh is mediated by a muscarinic receptor subtype most sensitive to 4-DAMP, while $I_{Ca}$ inhibition by high concentrations of ACh is via one preferring both 4-DAMP and oxybutynin, thus suggesting the involvement of muscarinic receptors showing similar pharmacological profiles to $M_3$ and $M_5$ receptors, respectively (see Discussion).

DISCUSSION

The major finding of the present study is that Z-338, a novel gastroprokinetic drug, which has been reported to inhibit pre-junctional muscarinic autoreceptors (2), enhances voltage-dependent L-type $Ca^{2+}$ current in gastric smooth muscle through stimulation of a muscarinic receptor showing biochemical and pharmacological properties consistent with those of the $M_5$ subtype. This is supported by several different lines of evidence. The effects of Z-338 are: i) resistant to PTX treatment, ii) almost completely eliminated by bath application of atropine or intracellular perfusion of GDPβS or PKC-selective inhibitory peptide, iii) mimicked by PDBu or low concentrations of ACh, both of which occurred in a non-additive fashion with respect to Z-338. These results strongly suggest the involvement of PI turnover-linked, G-protein coupled receptors; i.e., either $M_1$, $M_3$ or $M_5$ muscarinic receptor subtypes. iv) The observed pharmacological profile of the effects of Z-338 as well as ACh is most consistent with the reported profile of $M_3$ subtype; pirenzepine and AF-DX116 were ineffective, and oxybutynin showed a lower antagonistic efficacy than 4-DAMP against $I_{Ca}$ potentiation by Z-338 or ACh (Fig. 5; refs. 10, 11). Calculation with reported $pA_2$ values of muscarinic antagonists (see above) and an $ED_{50}$ value of Z-338 obtained in this study (0.12 μM, Fig. 2B) suggests that expected occupancy by Z-338 (1 μM) of $M_1$ and $M_3$ receptors in the presence of pirenzepine (1 μM) and AF-DX116 (1 μM), respectively, and of $M_3$ and $M_5$ receptors in the presence of 0.1 μM 4-DAMP is less than 10%, whereas that for $M_3$ and $M_5$ receptors with 0.1 μM oxybutynin is 14% and 63%, respectively. Notably, when 1 μM oxybutynin is present, the expected occupancy of $M_5$ receptor by Z-338 (1 μM) reduces to 17%, all values calculated being most compatible with the involvement of $M_5$-receptor. Thus, the most likely interpretation of these data is, although other nonspecific effects of Z-338 such as on G-protein and downstream effectors cannot entirely be precluded, that a muscarinic receptor subtype similar to $M_5$ subtype is functionally expressed in gastric smooth muscle and coupled specifically to voltage-dependent $Ca^{2+}$ channel via the $G_{q11}$/PKC pathway. On the other hand, inhibition of $I_{Ca}$ caused by high concentrations of ACh, which is deficient in the actions of Z-338, seems mediated by activation of another PI turnover-linked muscarinic receptor in gastric smooth muscle, most likely the $M_3$ subtype, since the inhibition was not affected by pirenzepine or AF-DX116, and comparably suppressed by 4-DAMP and oxybutynin. Inability of Z-338 to cause $I_{Ca}$ inhibition is compatible with the fact that no detectable binding occurs between tritiated Z-338 and $M_3$ muscarinic receptor (2).

Muscarinic receptors in the GI tract have been characterized mostly by means of radioligand binding techniques, and it has been reported that the predominant subtypes expressed in gut smooth muscles are $M_2$ and $M_3$ (12, 13). In contrast, in pre-junctional tissues in the GI tract such as myenteric neurones, $M_1$ and $M_2$ receptors are preponderant and thought to play primary roles as negative autoreceptors for ACh release (14, 15). It has been suggested that the $M_3$ subtype is functionally most important for mediating contractions in GI muscle, since potent $M_3$-receptor antagonists such as 4-DAMP strongly attenuate
peristaltic movements of whole gut and contractions of isolated muscle preparations induced neurally or by exogenously applied cholinomimetic drugs (12, 16). In contrast, despite its abundant expression, the extent of contribution of M3 receptor to excitatory responses in the GI tract remains rather elusive; only a limited role in antagonizing the relaxant effects of cAMP-increasing agents via inhibition of adenylate cyclase has been suggested (17). However, recent patch clamp experiments in guinea pig gastric and ileal smooth muscle cells revealed that M2 receptors also activate nonselective cation channels via a PTX-sensitive G12/13 G-protein, thereby depolarizing the membrane and in turn causing Ca2+ entry through the nifedipine-sensitive voltage-dependent pathway (18–20). Since these channels are very susceptible to changes in intracellular Ca2+ concentration (i.e., its elevation strongly potentiates the channel activities; 21, 22) as well as extracellular Ca2+ concentration (23), this M2 receptor-mediated mechanism may significantly contribute to generating maintained contractions in concert with IP3-mediated Ca2+ release caused by concomitant activation of M3 receptor (7). Significant contribution of M2 receptor mediated pathways to contractile responses of GI muscle has been clearly demonstrated in M2-receptor knock-out mice (13, 24).

Very recently, more modern biochemical and molecular strategies such as RT-PCR and immunocytochemical techniques have provided new lines of evidence for the distribution profile of muscarinic receptors in GI muscle. In both freshly dissociated and primary cultured human esophageal smooth muscle cells, mRNA transcripts for and plasma membrane-localized immunoreactivities to all muscarinic receptor subtypes including the M3 subtype were detected using their specific primers and antibodies (25, 26). The M3 receptor was originally found to be enriched in specialized regions of the brain, but later shown to be expressed more ubiquitously throughout the brain and also in non-neuronal tissues such as blood cells and salivary glands (10, 27). However, very little is yet known about what molecular targets and functions are associated with M3 receptors and their downstream signaling cascades, except for some indirect suggestions of their possible implications in modulating dopaminergic transmission and salivary gland secretion and activating nitric oxide synthase (10, 27). In this respect, our present results have provided the intriguing possibility that a muscarinic receptor pharmacologically indistinguishable from the M3 receptor upregulates L-type Ca2+ channel activities via activation of a specific PKC pathway and may contribute to enhancing the gastric motility (see below). Recently, modulation of L-type Ca2+ channels by muscarinic receptors was investigated by recombinantly expressing five subtypes of muscarinic receptors (m1 – m5) into an established fibroblast cell line, NIH 3T3 cells (28). Somewhat unexpectedly, stimulation of PI turnover-linked receptors, i.e., m1, m3 and m5 receptors by ACh all resulted in inhibition of Ca2+ currents via activation of PKC, thus seemingly contradicting the results of the present study. Although there is no good explanation for this discrepancy, it is worthwhile noting that in several native tissues, stimulation of PKC often biphasically regulates (29) or solely enhances (30 – 32) voltage-dependent Ca2+ channel activities. A possible molecular background for such differences may be the involvement of splice variants of αi/o subunit containing different PKC phosphorylation sites at the N-terminal (e.g., αiCa versus αi/Ca; 29, 33) or different PKC isoforms (34, 35). Obviously, further studies are required to distinguish between these possibilities.

A previous study employing recombinant expression of muscarinic receptors in Xenopus oocytes has shown that Z-338 is capable of binding M1 and M2 (but not M3) receptors and acts antagonistically on these receptors to inhibit activation of Ca2+-dependent Cl− currents by IP3-mediated Ca2+ release via M3 receptor and that of co-expressed GIRK1, an inward-rectifying K+ channel via the M3 receptor/PTX-insensitive G-protein pathway (2). These antagonistic effects of Z-338 are consistent with its observed enhancing actions on electrically evoked ACh release and excitatory junction potentials in guinea pig stomach muscle strips (2, 3), which can be accounted for by the inhibition of M1 and M2 autoreceptors residing in prejunctional cholinergic nerve terminals and negatively regulating ACh release (14, 36). In contrast, the data of the present study have clearly shown that Z-338 agonistically enhances L-type voltage-dependent Ca2+ channel activities probably through stimulation of post-junctional M3 receptor (see above), but cannot activate nonselective cation channels on the smooth muscle membrane even at high micromolar concentrations. The latter observation is consistent with the previous findings of microelectrode experiments that Z-338 (10 μM) did not alter the resting membrane potential or basal muscle tone (3), thus suggesting that Z-338 has no stimulatory effects on post-junctional M3 receptors. The lack of agonistic actions of Z-338 on post-junctional M3 receptors is also suggested by virtual absence of its inhibitory effects on L-type Ca2+ channels which are evident at high concentrations of ACh (see above). Such selective stimulation by Z-338 of post-junctional M3 but not M2 or M4 receptors may serve advantageously to specifically enhance spontaneous rhythmic contractions of GI muscle. It is well known that the rhythmic contractions are closely associated with nifedipine-sensitive voltage-dependent Ca2+ entry in smooth muscle cells which are activated by slow pace-making depolarizations (slow waves) originated from interstitial cells of Cajal (ICCs) (37, 38). Thus, if this Ca2+ entry would selectively be potentiated through stimulation
of M₁ receptor, only the rhythmic contractions would be accentuated with little accompanying increase in the basal tension. This might be therapeutically beneficial, because an excessive basal tone increase caused by M₂ and/or M₃ receptor stimulation might be deleterious rather than promoting to propel a bolus through the gut. Furthermore, the other additional actions of Z-338, e.g., enhancement of ACh release via inhibition of pre-junctional M₁ and M₂ autoreceptors as well as blockade of post-junctional M₂ receptor, would also help to more effectively amplify rhythmic contractions with little change in the resting tension.

Acknowledgments

We would like to thank Drs. H. Onoue and Y. Itonaga for pertinent advice in the early course of this work. Part of this work was financially supported by Zeria Pharmaceutical Co., Ltd. H.M. is a research fellow of the Japan Society for the Promotion of Science.

REFERENCES

1. Ueki S, Matsunaga Y, Matsuura T, Hori Y, Yoneta T, Kurimoto T, Tamaki H and Itoh Z: Z-338, a novel prokinetic agent, stimulates gastrointestinal motor activity and improves gatric emptying in the dog and rat. Naunyn Seminemedbergs Arch Pharmaco 358, Suppl 1, R351 (1998)
2. Ogishima M, Kaibara M, Ueki S, Kurimoto T and Taniyama K: Z-338 facilitates acetylcholine release from enteric neurons due to blockade of muscarinic autoreceptors in guinea pig stomach. J Pharmacol Exp Ther 294, 33 – 37 (2000)
3. Nakajima T, Nawata H and Ito Y: Z-338, a newly synthesized carboxyamide derivative, stimulates gastric motility through enhancing the excitatory neurotransmission. J Smooth Muscle Res 36, 69 – 81 (2000)
4. Morita H, Cousins H, Onoue H, Ito Y and Inoue R: Predominant distribution of nifedipine-insensitive, high voltage-activated Ca²⁺ channels in the terminal mesenteric terminal artery of guinea-pig. Circ Res 85, 596 – 605 (1999)
5. Katzka DA and Morad M: Properties of calcium channels in guinea-pig gastric myocyte. J Physiol (Lond) 413, 175 – 197 (1989)
6. Wade GR, Barbera J and Sims SM: Cholinergic inhibition of Ca²⁺ current in guinea-pig gastric and tracheal smooth muscle cells. J Physiol (Lond) 492, 307 – 319 (1996)
7. Bolton TB and Zholos AV: Activation of M₁ muscarinic receptors in guinea-pig ileum opens cationic channels modulated by M₁ muscarinic receptors. Life Sci 60, 1121 – 1128 (1997)
8. Kuriyama H, Kitamura K, Itoh T and Inoue R: Physiological features of visceral smooth muscle cells, with special reference to receptors and ion channels. Physiol Rev 78, 811 – 920 (1998)
9. Caulfield MP and Birdsall NJM: International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 50, 279 – 290 (1998)
10. Eglen RM and Nahorski SR: The muscarinic M₅ receptor: silent or emerging subtype? Br J Pharmacol 130, 13 – 21 (2000)
11. Watson N, Daniels DV, Ford APDW, Eglen RM and Hegde SS: Comparative pharmacology of recombinant human M3 and M5 muscarinic receptors expressed in CHO-K1 cells. Br J Pharmacol 127, 590 – 596 (1999)
12. Eglen, RM, Hedge, SS and Watson N: Muscarinic receptor subtypes and smooth muscle function. Pharmacol Rev 48, 531 – 565 (1996)
13. Eglen RM: Muscarinic receptors and gastrointestinal tract smooth muscle function. Life Sci 68, 2573 – 2578 (2001)
14. Kilbinger H, Dietrich C and von Bardeleben RS: Functional relevance of presynaptic muscarinic autoreceptors. J Physiol (Paris) 87, 77 – 81 (1993)
15. Ren J and Harty RF: Presynaptic muscarinic receptors modulate acetylcholine release from rat antral mucosa/submucosal nerves. Dig Dis Sci 39, 441 – 445 (1994)
16. Gilbert RJ and Dodds WJ: Effect of selective muscarinic antagonists on peristaltic contractions in opossum smooth muscle. Am J Physiol 250, G50 – G59 (1986)
17. Ehler RJ and Thomas EA: Functional role of M₂ muscarinic receptors in the guinea pig ileum. Life Sci 56, 965 – 971 (1995)
18. Inoue R and Isenberg G: Acetylcholine activates nonsensitive cation channels in guinea pig ileum through G protein. Am J Physiol (Cell Physiol) 258, C1173 – C1178 (1990)
19. Zholos AV and Bolton TB: Muscarinic receptor subtypes controlling the cationic current in guinea-pig ileal smooth muscle. Br J Pharmacol 122, 885 – 893 (1998)
20. Kim YC, Kim SJ, Sim JH, Cho CH, Juhan Y-S, Suh SH, So I and Kim KW: Suppression of the carbachol-activated non-selective cationic current by antibody against alpha subunit of G, protein in guinea-pig gastric myocytes. Pflugers Arch Eur J Physiol 436, 494 – 496 (1998)
21. Inoue R and Isenberg G: Intracellular calcium ions modulate acetylcholine-induced inward current in guinea-pig ileum. J Physiol (Lond) 424, 73 – 92 (1990)
22. Pacaud P and Bolton TB: Relation between muscarinic receptor cationic current and internal calcium in guinea-pig jejunal smooth muscle cells. J Physiol (Lond) 441, 477 – 499 (1991)
23. Kang TM, Kim YC, Sim JH, Rhee JC, Kim SJ, Uhm DY, So I and Kim KW: The properties of carbachol-activated non-selective cationic channels at the single channel level in guinea pig gastric myocytes. Jpn J Pharmacol 85, 291 – 298 (2001)
24. Matsu M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komita Y, Takahashi S and Taketo MM: Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M₃ subtype. Proc Natl Acad Sci USA 97, 9579 – 9584 (2000)
25. Preiksaitis HG, Kryski PS, Chrones T, Rajgopal V and Laurier LG: Pharmacological and molecular characterization of muscarinic receptor subtypes in human esophageal smooth muscle. J Pharmacol Exp Ther 295, 879 – 888 (2000)
26. Wang J, Kryski PS, Laurier LG, Sims SM and Preiksaitis HG: Human esophageal smooth muscle cells express muscarinic receptor subtypes M₁ through M₃. Am J Physiol (Gastrointest Liver Physiol) 279, G1059 – G1069 (2000)
27. Reeve CM, Ferrari-Dileo G and Flynn DD: The M₅ (m5) receptor subtype: fact or fiction? Life Sci 60, 1105 – 1112 (1997)
28. Pemberton KE and Jones SVP: Inhibition of the L-type calcium channel by the five muscarinic receptors (m1 – m5) expressed in NIH 3T3 cells. Pflugers Arch Eur J Physiol 433, 505 – 514 (1997)
29. Hofmann F, Lacinova L and Klugbauer: Voltage-dependent calcium channels: from structure to function. Rev Physiol Biochem Pharmacol 139, 33 – 87 (1999)
Clapp LH, Michel B, Vivaudou JV, Walsh JV Jr and Singer JJ: Acetylcholine increases voltage-activated Ca\textsuperscript{2+} current in freshly dissociated smooth muscle. Proc Natl Acad Sci USA 84, 2092 – 2096 (1987)

Fish RD, Sperti G, Colucci WS and Clapham DE: Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. Circ Res 62, 1049 – 1054 (1987)

Vivaudou MB, Clapp LH, Walsh, JV Jr and Singer JJ: Regulation of one type of Ca\textsuperscript{2+} current in smooth muscle cells by diacylglycerol and acetylcholine. FASEB J 2, 2497 – 2504 (1988)

McHugh D, Sharp EM, Scheuer T and Catterall WA: Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. Proc Natl Acad Sci USA 97, 12334 – 12338 (2000)

Hu K, Mochly-Rosen D and Boutjdir M: Evidence for functional role of εPKC isozyme in the regulation of cardiac Ca\textsuperscript{2+} channels. Am J Physiol (Heart Circ Physiol) 279, H2658 – H2664 (2000)

Way KJ, Chou E and King GL: Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends Pharmacol Sci 21, 181 – 187 (2000)

Starke K, Göthert M and Kilbinger H: Modulation of neurotransmitter release by presynaptic autoreceptors. Physiol Rev 69, 864 – 989 (1989)

Huizinga JD, Thuneberg L, Vanderwinden J-M and Rumessen JJ: Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal motor disorders. Trends Pharmacol Sci 18, 393 – 403 (1997)

Horowitz B, Ward S and Sanders KM: Cellular and molecular basis for electrical rhythmicity in gastrointestinal muscles. Annu Rev Physiol 61, 19 – 43 (1999)