Structure–Activity Study of Intact Porcine Motilin

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Biologically important sites on intact porcine motilin (pMTL) were explored using its partial peptides. The partial peptides were synthesized using Fmoc (9-fluorenylmethyloxycarbonyl) solid phase methodology, and tested for the binding activity to motilin receptor and the smooth muscle contractile activity. The results were as follows: important residues for the contractile activity were found to be Phe[1], Ile[4], and Tyr[7], and an open space existed beyond the N-terminus between motilin and its receptor. On the model of interaction between motilin and motilin receptor evolved from these results, the three points of interaction, due to Phe[1], Ile[4], and Tyr[7], and the presence of an open space were expected. The motilin agonist and antagonist, designed on this model, will help the inquiry into motilin associated diseases.

Key words motilin; structure–activity relationship; partial peptide

Motilin, a single chain peptide of 22 amino acid residues, was isolated from endocrine cells in gastrointestinal mucosa of various species.[1)] Although the biological function of motilin has not been fully identified, it was reported to stimulate gastrointestinal motility in many species.[1)]

The amino acid sequence of porcine motilin (pMTL) was first identified in 1973 (FVPIF TYGEL QRMQE KERNK GQ).[2,3)] Human motilin, identified in 1985, was found to be identical to that of porcine, but different from canine by five residues at the seventh, eighth, 12th, 13th, and 14th.[1,4)]

The physiological relevance of motilin to some gastrointestinal symptoms was suggested: early satiety, abdominal distention, nausea, vomiting, and anorexia.[3)] Although discovery of pure motilin agonist and antagonist is required to investigate the biological and physiological mechanism of the peptide and for treatment of patients with its associated diseases, structure–activity relationship (SAR) analyses on intact motilin have not been sufficient to achieve an effective and prospective design of motilin agonist and antagonist.

In an early study on the SAR of pMTL, the following limited information was reported: partial peptide, pMTL(1—15) had the one half activity *in vitro*, compared with the full-length peptide using rabbit smooth muscle.[5)] In addition, short peptide pMTL(1—8) and N-terminus deleted peptide pMTL(6—22) possessed only 0.1% and 2%, respectively, of the activity of the full-length peptide, and C-terminal peptides pMTL(9—22) and pMTL(14—22) were inactive.[5—8)] The SAR concerning (Leu[13])pMTL and its partial peptides, in which methionine residue was substituted by leucine residue, was reported by Galdes and co-workers.[9,10)] Although methionine is usually categorized as an uncommon hydrophobic amino acid, an essential hydrogen bond of the sulfur atom of methionine was observed on many peptides and proteins.[11—14)] The SAR concerning intact pMTL had not been studied.

Therefore, we synthesized a considerable number of partial peptides of intact pMTL in order to complete the SAR analyses. We would like, herein, to report the critical moiety for the activity of intact pMTL and the model of the pMTL binding with its receptor.

Chemistry The partial peptides were synthesized (Table 1) in a solid phase methodology,[15,16)] according to an N^{α}-Fmoc (N^{α}-9-fluorenylmethyloxycarbonyl) group strategy on a MBHA-resin (*p*-methylbenzhydrylamine substituted polystyrene resin).[17,18)] The deprotection of N^{α}-Fmoc group was conducted with 20% piperidine in *N*,*N*-dimethylformamide (DMF) solution prior to coupling with the next protected amino acid. All amino acids esterified with pentafluorophenol (Pfp) were coupled in the presence of *N*-hydroxybenzotriazole (HOBt). Threonine esterified with 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazole (Dhbt) was coupled without additives. The completion of the couplings was verified by the Kaiser test.[19)] The peptides were simultaneously deprotected and cleaved from the resin by treatments with trifluoroacetic acid (TFA)/trimethylsilyltrifrate (TMSOTf)/*m*-cresol/thioanisole (100 : 20 : 5.23 : 1.7; v/v) for 1—2 h at 0 °C or room temperature varied with the side chain protecting groups.

Results and Discussion

The synthesized partial peptides of intact pMTL were tested for the binding activity to motilin receptor and the smooth muscle contractile activity in rabbit smooth muscle (Table 2). The binding activity and the contractile activity mostly correlated on all the partial peptides.

C-Terminus and N-Terminus Deletion Study pMTL (1—16)-NH_2 (**1**) and pMTL(1—14)-NH_2 (**2**) had the strong contractile activity with EC_{50} of 37.8 and 41.1 nM, respectively, slightly lower than that of intact pMTL. pMTL(1—12)-NH_2 (**3**) still had the contractile activity with EC_{50} of 69 nM. Although pMTL(1—10)-NH_2 (**4**) showed the binding activity with IC_{50} of 13 nM, contractile activity was low (EC_{50}: 413 nM). pMTL(1—8)-NH_2 (**5**) had very weak binding activity with IC_{50} of 4080 nM and contractile activity with EC_{50} of 62100 nM. For the N-terminus, all of the partial peptides that had deleted N-terminus (**6**—**12**) lost the activity.

These results strongly suggested that 10 amino acid residues of N-terminus were critical, and C-terminal residues after the 16th amino acid were not so important for the activity.

N-terminus and Open Space The addition of Ala to N-terminus of pMTL(1—10)-NH_2 (**14**) still had the activity with EC_{50} of 426 nM, suggesting that there existed the open space between motilin and its receptor beyond N-terminus.

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Table 1. Primary Structures, Abbreviated Names, and Analytical Properties of Intact pMTL and Partial Peptides

No.	Abbreviated names	Primary structures	HPLC t_R (min), (purity,%)[a]	FAB-MS (MH^+), (Calcd)[b]
—	pMTL	FVPIFTYGELQRMQEKERNKGQ	—	—
1	pMTL(1—16)-NH_2	FVPIFTYGELQRMQEK-NH_2	18.52 (92)	n.d.
2	pMTL(1—14)-NH_2	FVPIFTYGELQRMQ-NH_2	19.1 (100)	n.d.
3	pMTL(1—12)-NH_2	FVPIFTYGELQR-NH_2	17.97 (100)	n.d.
4	pMTL(1—10)-NH_2	FVPIFTYGEL-NH_2	18.3 (100)	1184 (1184.6354)
5	pMTL(1—8)-NH_2	FVPIFTYG-NH_2	16.78 (100)	n.d.
6	pMTL(2—12)-NH_2	VPIFTYGELQR-NH_2	19.80 (96)	n.d.
7	pMTL(3—12)-NH_2	PIFTYGELQR-NH_2	20.87 (96)	n.d.
8	pMTL(4—12)-NH_2	IFTYGELQR-NH_2	20.28 (94)	n.d.
9	pMTL(5—12)-NH_2	FTYGELQR-NH_2	18.17 (95)	n.d.
10	pMTL(6—12)-NH_2	TYGELQR-NH_2	16.46 (91)	n.d.
11	pMTL(6—22)	TYGELQRMQEKERNKGQ	17.72 (97)	n.d.
12	pMTL(2—10)-NH_2	VPIFTYGEL-NH_2	14.77 (98)	1037.5668 (1037.5671)
13	[D-Phe[1]]pMTL(1—10)-NH_2	D-Phe-VPIFTYGEL-NH_2	17.63 (98)	1184.6357 (1184.6355)
14	Ala-pMTL(1—10)-NH_2	Ala-FVPIFTYGEL-NH_2	16.63 (97)	1255.6735 (1255.6726)
15	[Ala[1]]pMTL(1—12)-NH_2	AVPIFTYGELQR-NH_2	14.03 (95)	1392.7638 (1392.7638)
16	[Ala[2]]pMTL(1—12)-NH_2	FAPIFTYGELQR-NH_2	14.79 (96)	1440.7654 (1440.7638)
17	[Ala[3]]pMTL(1—12)-NH_2	FVAIFTYGELQR-NH_2	15.05 (97)	1442.7785 (1442.7795)
18	[Ala[4]]pMTL(1—12)-NH_2	FVPAFTYGELQR-NH_2	13.87 (98)	1426.7474 (1426.7482)
19	[Ala[5]]pMTL(1—12)-NH_2	FVPIATYGELQR-NH_2	13.28 (100)	1392.7644 (1392.7638)
20	[Ala[6]]pMTL(1—12)-NH_2	FVPIFAYGELQR-NH_2	15.49 (98)	1438.7860 (1438.7845)
21	[Ala[7]]pMTL(1—12)-NH_2	FVPIFTAGELQR-NH_2	14.68 (98)	1376.7683 (1376.7689)
22	[Ala[8]]pMTL(1—12)-NH_2	FVPIFTYAELQR-NH_2	15.64 (97)	1482.8118 (1482.8108)
23	[Ala[9]]pMTL(1—12)-NH_2	FVPIFTYGALQR-NH_2	15.28 (97)	1410.7897 (1410.7896)
24	[Ala[10]]pMTL(1—12)-NH_2	FVPIFTYGEAQR-NH_2	14.28 (98)	1426.7488 (1426.7482)

a) 100 : 0 to 30 : 70 (0.1% aqueous TFA: 0.1% TFA in acetonitrile) over 25 min, 1.0 ml/min. *b*) n.d. means not determined.

Table 2. Abbreviated Names, Contractile Activity and Binding Activity of Intact pMTL and Partial Peptides

No.	Abbreviated names	Contractile activity (EC_{50}: nM)[a]	Binding activity (IC_{50}: nM)[b]
—	pMTL	5.0±2.2 (3)	1.0
1	pMTL(1—16)-NH_2	37.8±1.4 (3)	17
2	pMTL(1—14)-NH_2	41.1±10.1 (3)	5.9
3	pMTL(1—12)-NH_2	69.0±39.8 (3)	2.1
4	pMTL(1—10)-NH_2	413±79.2 (4)	13
5	pMTL(1—8)-NH_2	62100±15000 (6)	4080±1400[c]
6	pMTL(2—12)-NH_2	>100000	3500
7	pMTL(3—12)-NH_2	>100000	3200
8	pMTL(4—12)-NH_2	>100000	17000
9	pMTL(5—12)-NH_2	>100000	>100000
10	pMTL(6—12)-NH_2	>100000	>1000000
11	pMTL(6—22)	>100000	>1000000
12	pMTL(2—10)-NH_2	>100000	>100000
13	[D-Phe[1]]pMTL(1—10)-NH_2	4050±541 (3)	570
14	Ala-pMTL(1—10)-NH_2	426±46.7 (4)	36
15	[Ala[1]]pMTL(1—12)-NH_2	54800±31500 (6)	170
16	[Ala[2]]pMTL(1—12)-NH_2	4780±1200 (4)	29
17	[Ala[3]]pMTL(1—12)-NH_2	26.0±6.8 (7)	0.38
18	[Ala[4]]pMTL(1—12)-NH_2	49400±514200 (4)	1200
19	[Ala[5]]pMTL(1—12)-NH_2	2470±1575 (4)	48
20	[Ala[6]]pMTL(1—12)-NH_2	2380±566(6)	160
21	[Ala[7]]pMTL(1—12)-NH_2	11300±5780 (6)	360
22	[Ala[8]]pMTL(1—12)-NH_2	557±308 (4)	16
23	[Ala[9]]pMTL(1—12)-NH_2	1170±1000 (5)	130
24	[Ala[10]]pMTL(1—12)-NH_2	244±96.2 (4)	10

a) Contractile activity data are presented as the mean±S.D. with the number of repetitions from independent assays (*n*) listed in parentheses. *b*) Binding activity data are presented from one or two determination(s), unless otherwise noted. *c*) Mean±S.D., $n=4$ determinations.

Generally, an amino group, which possesses cationic charge under the physiological pH, often contributes to strong ionic interaction with protein on many peptide–receptor interactions.[20] Thus, the charged amino group of N-terminus of pMTL had been expected to play a role in the construction of an active conformation. However, surprisingly, the contractile activity and the binding activity were not lost after N-terminus acylated by additional Ala in Ala–pMTL(1—10)-NH_2 (**14**). This fact suggested that the amino moiety of Phe1 was not important for the activity.

Ala Scan Study Ala scan was studied in pMTL(1—12)-NH_2 which had sufficient activity to survey the contribution

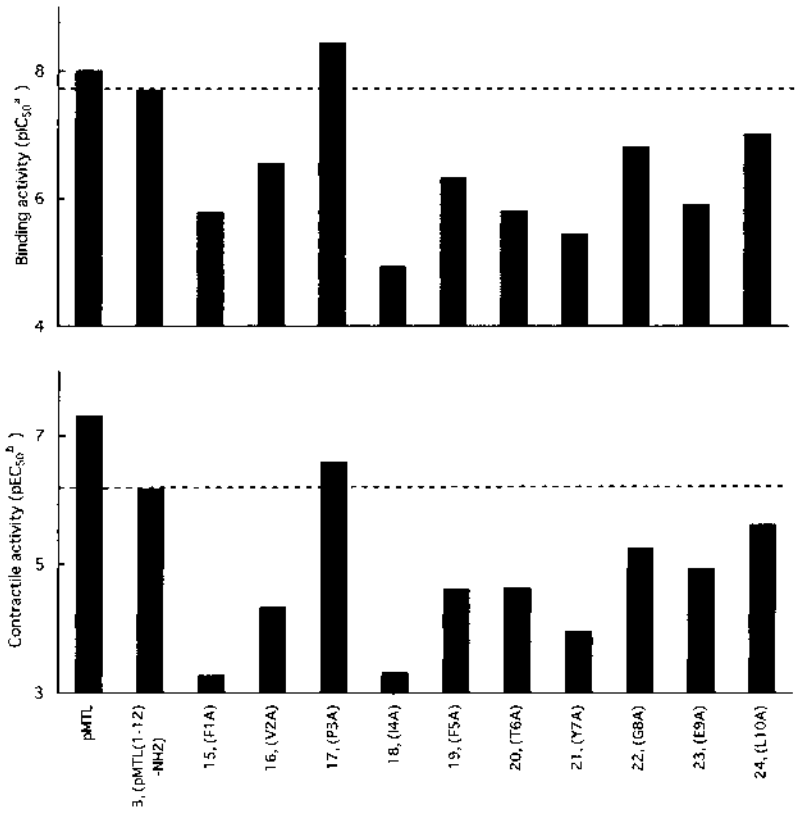

Fig. 1. Ala Scan of pMTL(1—12)-NH_2

a) pIC_{50} means $-\log(IC_{50})$. *b*) pEC_{50} means $-\log(EC_{50})$.

of each residue to the activity and to avoid the influence of Met^{13} (Table 1, Fig. 1). In the Ala scan of pMTL(1—12)-NH_2, the resulting peptides exhibited the agonistic effect to varying extents: [Ala^1]pMTL(1—12)-NH_2 (**15**), [Ala^4]pMTL(1—12)-NH_2 (**18**), and [Ala^7]pMTL(1—12)-NH_2 (**21**) were much less effective, whereas [Ala^8]pMTL(1—12)-NH_2 (**22**) and [Ala^{10}]pMTL(1—12)-NH_2 (**24**) retained the contractile activity (Table 2). These results suggested that Phe^1, Ile^4, and Tyr^7 were important active sites. Although [Ala^2]pMTL(1—12)-NH_2 (**16**), [Ala^5]pMTL(1—12)-NH_2 (**19**), [Ala^6]pMTL(1—12)-NH_2 (**20**), and [Ala^9]pMTL(1—12)-NH_2 (**23**) showed low affinity on binding assay, the contractile activity of these peptides was better than that of compounds **15**, **18**, and **21**. Therefore, it was assumed that Phe^1, Ile^4 and Tyr^7 were important for both binding and contractile activity, and that Val^2, Phe^5, Thr^6 and Glu^9 contributed mainly to binding.

[Ala^3]pMTL(1—12)-NH_2 (**17**), compared with pMTL(1—12)-NH_2 (**3**), was 6 times and 3 times more effective in binding activity and in contractile activity, respectively. Especially, the binding activity was higher than that of intact pMTL. This surprising fact suggested that the rigid structure of Pro^3 of intact motilin would not fit the best conformation for the binding with motilin receptor.

Although no crystal structure for motilin has been reported, three dimensional structures of motilin in liquid phase were presented by means of ^{1}H-NMR studies by several institutes.[21—24] These reports revealed that the structure of the C-terminal side from the residue Glu^9 to Lys^{20} of motilin was folded into an α-helix and the residues from Pro^3 to Thr^6 formed a wide turn, and these two segments were connected by a "hinge-like" region around Tyr^7. Especially, it

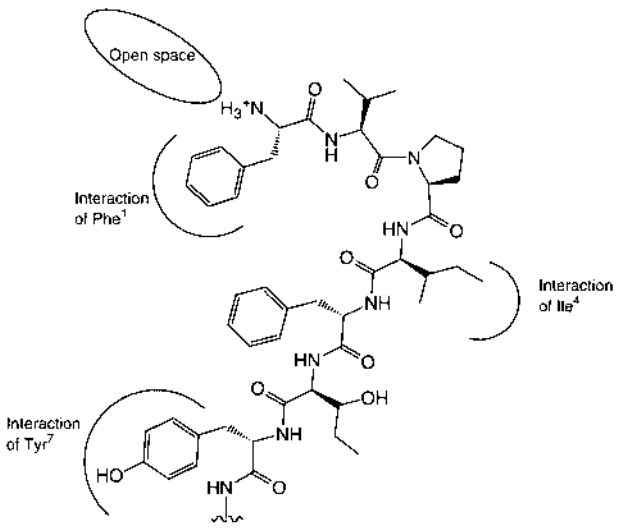

Fig. 2. Model of the Recognition between pMTL and Motilin Receptor

was reported that the Val^2–Pro^3 peptide bond was *trans* due to a strong nuclear Overhauser enhancement (NOE).[21] This trans Val^2–Pro^3 peptide bond led to a comparatively rigid turn structure which was not identical with the best conformation to contractile and binding activity. Replacement of Pro^3 to Ala could permit the structure to change from the rigid turn to a more active conformation.

Our result suggested that the critical structure for activity (Phe^1, Ile^4, Tyr^7) was located in this N-terminal part that consisted of the wide turn and hinge-like region, outside the α-helix region. These results almost corresponded to those

from (Leu[13])MTL.[9,10] Therefore Met[13] was thought to act merely as a hydrophobic amino acid similar to Leu, and the α-helix region containing Met[13] was not necessary for the activity.

Model of Interaction between Motilin and Motilin Receptor From the results obtained in this study, we developed a model (Fig. 2) of the motilin binding with its receptor in order to understand the detailed interaction. In this model, intact pMTL would have interactions *via* three points toward its receptor: Phe[1], Ile[4], and Tyr[7]. These interactions were thought to be necessary for tight binding between motilin and its receptor. Interaction of Phe[1] and Ile[4], which were categorized as hydrophobic amino acids, was expected to be hydrophobic. On Tyr[7], there was a possibility of both ionic and hydrophobic interaction. In addition, interaction in the open space was expected to contribute to the binding. Detailed features of the open space will be announced in a subsequent report. The ionic interaction of N-terminus, which formed in intact motilin, did not contribute to the binding.

Conclusion

We have synthesized the partial peptides of intact pMTL, and explored the SARs of this peptide.

In the model evolved from the results of this research, three points of interaction (Phe[1], Ile[4], and Tyr[7]) were critical. However, the ionic interaction of N-terminus was not important for the binding between motilin and its receptor.

The compounds with these interactions are considered to have high affinity to the motilin receptor, leading to potent motilin agonist and antagonist. Using this agonist and antagonist the biological and physiological mechanism of motilin will be investigated, and this will assist in developing a remedy against motilin associated diseases.

Experimental

Materials N^{α}-Fmoc amino acid, Pfp esters, and Dhbt ester were purchased from either MilliGen/Biosearch (Burlington, MA) or Watanabe Chemical (Japan). MBHA-resin (NH_2: 0.38 mmol/g) was purchased from the Peptide Institute (Japan). TFA, TMSOTf, 1,3-diisopropylcarbodiimide (DIC), and HOBt were purchased from Watanabe Chemical. *m*-Cresol, and thioanisole were purchased from Tokyo Chemical Industry (Japan). DMF was purchased from Kokusan Chemical (Japan). HPLC grade acetonitrile, and water were from Kanto Chemical (Japan) or Junsei Chemical (Japan).

Solid Phase Peptide Synthesis of pMTL(1—12)-NH_2 (3) MBHA-resin (NH_2: 0.38 mmol/g; 2.616 g) was treated twice with 20% piperidine in DMF and washed eight times with DMF. N^{α}-Fmoc-Arg(Mtr)-OH (4 eq) was then added followed by HOBt (4 eq) and DIC (4 eq). The coupling reaction mixture was shaken on a manual shaker for 18 h at room temperature. The resin was then washed six times with DMF. The following N^{α}-Fmoc amino acid Pfp esters and Dhbt ester were sequentially coupled to a growing peptide chain: N^{α}-Fmoc-Gln(Trt)-OPfp, N^{α}-Fmoc-Leu-OPfp, N^{α}-Fmoc-Glu(Ot-butyl)-OPfp, N^{α}-Fmoc-Gly-OPfp, N^{α}-Fmoc-Tyr(t-butyl)-OPfp, N^{α}-Fmoc-Thr(t-butyl)-ODhbt, N^{α}-Fmoc-Phe-OPfp, N^{α}-Fmoc-Ile-OPfp, N^{α}-Fmoc-Pro-OPfp, N^{α}-Fmoc-Val-OPfp, N^{α}-Fmoc-Phe-OPfp. Each coupling cycle consisted of the following: (1) treat twice with 20% piperidine in DMF for 30 min each time, (2) wash eight times with DMF, (3) couple the amino acid active ester, (4) wash six times with DMF. The coupling of the last amino acid was as described above, but additional steps were added as follows: (5) treat twice with 20% piperidine in DMF for 30 min each time, (6) wash eight times with DMF. All the N^{α}-Fmoc amino acid Pfp esters (4 eq) were coupled to a growing peptide chain using HOBt (4 eq) in DMF. N^{α}-Fmoc-Thr(t-butyl)-ODhbt (4 eq) was coupled in DMF. All the coupling reactions were shaken on a manual shaker for 4—48 h at room temperature. After deprotection of the last N^{α}-Fmoc group, the peptide resin was washed with methanol and dried *in vacuo* to yield the protected pMTL(1—12)-NH_2-resin.

The protected peptide-resin was treated with TFA/TMSOTf/*m*-cresol/thioanisole (100 : 20 : 5.23 : 1.7; v/v) for 1 h at 0 °C and an additional hour at room temperature. The solution was filtered and added dropwise to excess cold diethyl ether. The precipitated peptide was filtered, washed with diethyl ether, and dried. The crude peptide was purified by preparative HPLC with a linear gradient of 0—60% 0.1% TFA in acetonitrile against 0.1% aqueous TFA, over 60 min, at 10 ml/min. The lyophilized product was a white amorphous solid. Overall yield: 46%.

A similar protocol was carried out for the syntheses of the peptides in this manuscript. The side chain protections of the other amino acids were Arg(Mtr), Asn(Trt) and Lys(Boc).

Peptide Purification The crude peptides were purified by RP-HPLC using a Waters semi-prep system with C18 YMC-Pack S-343-15 (YMC, Japan), 15 μm, 120 Å, 20×250 mm, eluting with a linear acetonitrile gradient (0—60%) in water containing a constant concentration of TFA (0.1%, v/v) over 60 min at a flow rate of 10 ml/min. The peptide fractions which were purified by analytical HPLC were lyophilized, and the powder was kept at −20 °C until their biological assay.

Peptide Analysis The purity of the final products was confirmed by RP-HPLC using a Beckman analytical HPLC system with an analytical column (YMC-Pack A-302, 4.6×150 mm, 120 Å, 5 μm particle size, YMC, Japan). The gradient for analytical RP-HPLC was as follows: 100 : 0 to 30 : 70 (0.1% aqueous TFA/0.1% TFA in acetonitrile) over 25 min at 1 ml/min. Recording and quantification were accomplished with a Waters chromatographic data module (Waters model 741).

The composition of the peptide was verified by quantitative amino acid analysis using Pico-Tag methodology (Waters) and phenyl isothiocyanate (PITC) as amino acid derivatization reagents. Lyophilized samples of peptides (50—1000 pmol) were placed in heat-treated borosilicated tubes (50×4 mm), sealed, and hydrolyzed with 200 μl of 6 N HCl containing 1% phenol in the Pico-Tag workstation for 1 h at 150 °C. A Pico-Tag column (15×3.9 mm) was employed to separate the PITC-amino acid derivatives. The composition of the compounds was as expected.

The molecular weight of the compounds was determined by FAB-MS (VG11-250, VG Analytical, UK). The values are expressed as MH^+.

Receptor Binding Assay Binding assay for motilin receptor was performed according to the procedure introduced by Bormans *et al*.[25] with a slight modification. After exsanguination, the upper part of intestine (about 50 cm) of the rabbit was rapidly removed and rinsed with ice cold 0.9% saline. The smooth muscle tissue was dissected free from connective tissue and mucosa, finely minced and homogenized in 50 mM Tris–HCl buffer (pH=7.4) at 0 °C using tapered tissue grinders (Wheaten, Milliville, NJ) at 2000 rpm for 30 s. The homogenate was centrifuged at 1500×***g*** for 5 min and was washed twice with a fresh buffer. The final pellet was resuspended in 50 ml of 50 mM Tris–HCl buffer (pH=8.0, containing 10 mM $MgCl_2$, 1.5% bovine serum albumin) for binding studies. The protein concentration was determined by the method of Lowry *et al*.[26] using bovine serum albumin as the standard.

The homogenate (about 1.0 mg protein/assay) was incubated at 25 °C for 120 min with 25 pM ^{125}I-pMTL (specific activity, 33—66 kBq/pmol); the final volume was 1 ml. After incubation, the reaction was stopped by adding 2 ml of cold buffer. Bound and free reagents were separated by centrifugation at 1500×***g*** for 5 min. The pellet was washed with a cold buffer, and its radioactivity was determined with a gamma counter (ARC-300, Aroka, Tokyo, Japan). The concentration displacing 50% of the label is expressed (IC_{50}).

Contraction Assay Male Japanese-white rabbits (about 3 kg) were used. The animals were anesthetized with thiopental sodium (30 mg/kg, i.v.) and exsanguinated. The upper part of the small intestine was rapidly removed after laparotomy and placed in an ice-cold modified Krebs' solution composed of (in mM): NaCl 120.0, KCl 4.7, $CaCl_2$ 2.4, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 24.5 and glucose 5.6 (pH=7.4). The duodenum was washed, freed from mesenteric attachment and cut along the longitudinal axis to obtain muscle strips approximately 10 mm long and 3 mm wide. The preparation was mounted in an organ bath containing 10 ml of modified Krebs' solution kept at 28 °C to prevent excessive spontaneous contractions.[27] The solution was gassed with a mixture of 95% O_2 and 5% CO_2. The longitudinal strips were initially loaded with a 1.0-g weight, and contractile activity was measured by means of isotonic transducers (ME-4012, Medical Electronics Co., Tokyo) and recorded on an ink-writing recorder (Type 3066, Yanagisawa-Denki, Tokyo). Before the experiments, each strip was subjected to repeated stimulation with 100 μM ACh until a reproducible response was obtained. The contractile potency of each compound was expressed as a percentage of that induced by 100 μM ACh. The dose giving 50% of the response is expressed (EC_{50}).

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