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N-TERMINAL PORTION OF MOTILIN DETERMINES ITS BIOLOGICAL ACTIVITY

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This study aimed to identify the portion of the 22 amino acid sequence of motilin responsible for the biological activity of the peptide. The contraction of rabbit duodenal muscle *in vitro* was measured when exposed to synthetic fragments of motilin corresponding to various sequences of the C- or N-terminal portions of the molecule. Fragments 2-22 or 3-22 (where the initial amino acids of the N-terminal ending were removed) were more than 1000 times less potent than the native molecule 1-22. Fragment 1-9 (where the last 13 amino acids located at the C-terminal side of motilin were removed) was devoid of any contractile capacity, while synthetic fragments whose C-terminal structure extended beyond the 1-9 motilin sequence maintained almost complete biological activity. N-terminal amino acid sequence 1-9 is therefore an essential determinant of the contractile activity of motilin.

Motilin is a 22 amino acid polypeptide stimulating the contraction of the intestinal smooth muscle(1). Synthesized in endocrine cells of the duodeno-jejunal mucosa, motilin circulates in the blood of numerous mammals to regulate the phasic motor activity, the migrating motor complex (MMC), of the fasting stomach and small intestine. In the dog, experimental evidence revealed a physiological role for plasma motilin as an inducer of the phase III, the peristaltic contractile activity of the MMC (2,3).

The structure-activity characteristics of motilin are still unclear. Most information on this topic was obtained by studying in vitro the contraction of longitudinal smooth muscles from rabbit duodenum in response to motilin or motilin analogues. Synthetic analogue peptides with amino acids substitutions in the middle portion or the molecule [position 7, 8, 12 (4), 13, 14 (4,5) or 15 (6)] expressed bioactivity identical to the native molecule. Synthetic C-terminal fragments such as motilin 17-22, 14-22, 9-22 or 6-22 (7,8) were all inactive to induce muscle contraction, suggesting biological activity resided in the N-terminal portion of the molecule. Substitution of Phe in position 1 by Ser or Lys dramatically decreased the contractile activity of motilin (9), supporting furthermore the

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biological importance of the N-terminal portion. However, N-terminal fragments such as motilin 1-5 (7), 1-6 (10) or even 1-16 (11) appeared only weakly effective and forced the conclusion that the biological activity of motilin relied on a major portion of the molecule.

This study was designed to elucidate the portion of the motilin sequence responsible for its biological activity.

METHODS

Peptide synthesis:

Motilin and motilin fragments were assembled by the solid phase synthesis method (12), using Boc (13) chemistry. Benzhydrylamine or methylbenzhydrylamine resin supports were used in order to obtain directly C-terminal amide for fragments or motilin glutamine residue (from precursor alpha-benzyl glutamyl) following HF cleavage. Ten syntheses could be carried out simultaneously on a 1 mmol scale (2.5 g resin) using a homemade, nitrogen-scrubbed multireactor synthesizer. Boc protection was removed by means of 40% (v/v) TFA in CH_2Cl_2, followed by usual CH_2Cl_2 and DMF wash cycles. DIEA (5% v/v) in CH_2Cl_2 was used to displace the TFA salt. Couplings of protected amino acid intermediate were mediated by means of BOP reagent (14). Completion of coupling (99%+) was monitored by means of the Kaiser test (15). Simultaneous clevage from the resin, formation of C-terminal amide and removal of side chain protecting groups were performed in one operation with liquid HF (90 min, 0°C) in the presence of scavengers (ethanethiol, m-cresol). After evaporating HF in vacuo and washing the resin with ethyl ether, each peptide was extracted from the resin with TFA (20 ml/g). TFA was rapidly evaporated in vacuo and the peptide precipitated with ethyl ether, filtered and dried in the presence of K0H pellets. Yields of crude peptide were nearly quantitative, as calculated from the original substitution of resin. Purity of crude peptides varied between 35 and 75%, as estimated by analytical HPLC patterns. All motilin fragments were obtained with a purity superior to 98% (evaluated by analytical HPLC using a 5 micron C_{18} Pharmacia column and TFA/acetonitrile), following a single purification step by preparative HPLC (Vydac C_{18}, 15-20 micron, 4 x 60 cm column, Waters Delta Prep) in 0.1% TFA/acetonitrile gradients. Authenticity of synthetic peptides was confirmed by amino acid analysis and FAB mass spectrometry.

Muscle contraction:

Muscle contractile activity was evaluated in vitro with a muscle strip bioassay system. Rabbits were anesthetized with thiopental before laparotomy and death. The duodenal wall was exposed and cut along its longitudinal axis in strips of 0.3 x 15 mm. After removal of the mucosa by blunt dissection, each muscle strip was suspended in an organ bath filled with an oxygenated (95% 0_2-5% $C0_2$) Krebs-Ringer solution. Muscular contraction was evaluated by a force transducer (model FC.03, Grass Instrument Co., Quincy, Mass.) connected to a recorder (Model R611, Beckman Instruments, Fullerton, Calif.). The bioactivity of the fragments was tested on strips exposed to increasing concentrations (10^{-13} to 10^{-6} M) of synthetic motilin or fragments and to acetylcholine 10^{-5} M. The contractile response was evaluated by measuring the maximal vertical displacement induced by peptides and expressing it as the percentage of maximal response to acetylcholine. The amount of peptide require to elicitate a contraction equivalent to 50% of the maximal reponse to acetylcholine was determined (ED50).

To evaluated the relative participation of the N-terminal and the C-terminal portions of the peptide in its contractile effect, the strategy was to verify the action of motilin

Table 1. Biological activity of C-terminal fragments

Motilin analogues	ED_{50} ± SE (nM)
1-22	3 ± 1
2-22	> 10,000
3-22	> 10,000

fragments where successive amino acids of the original sequence would be progressively removed or from the C-terminal or from the N-terminal ending until significant loss in the bioactivity was obtained.

RESULTS

Structural alterations in the N-terminal segment of the peptide induced marked alterations of its bioactivity. Removal of the first or the first two amino acids decreased by more than 1000 times the bioactivity of the molecule. The results obtained with the C-terminal fragments are shown on table 1.

When amino acids were successively removed from the C-terminal ending, the full capacity of the peptide to induce the muscle contraction was maintained until the 10 last amino acids were discarded. Motilin 1-11 and 1-10 showed slightly decreased activity and it is only with the fragment 1-9 that an obvious loss of bioactivity could be detected. The results obtained with the N-terminal fragments are shown on table 2.

Figure 1 shows the muscle contraction obtained in response to increasing doses of motilin 1-22, 1-12, 2-22 and 1-9.

CONCLUSIONS

Our study clearly shows that the bioactive portion of the motilin molecule resides in the N-terminal ending. From the initial studies done with motilin, the portion of the motilin

Table 2. Biological activity of N-terminal fragments

Motilin analogues	ED_{50} ± SE (nM)
1-22	3 ± 1
1-21	17 ± 15
1-20	36 ± 21
1-19	14 ± 8
1-15	1 ± 0.4
1-12	6 ± 3
1-11	641 ± 140
1-10	252 ± 198
1-9	> 10,000

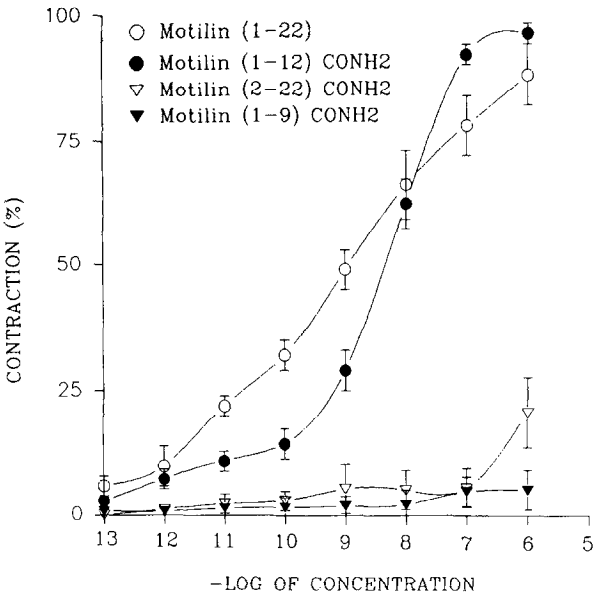

Figure 1. Dose-response curves of motilin 1-22 and of various motilin fragments. The muscle contraction obtained is expressed on the ordinate as the percentage of the maximal response induced by acetylcholine 10^{-5} M. n≥5 for each peptide.

sequence responsible for its bioactivity remained an enigma. Indeed, in these studies, neither C-terminal fragments such as motilin 6-22 (8), nor N-terminal fragments as long as the 1-16 analogue (11) could express a contractile activity comparable to the one of the native 1-22 peptide. A study published later by Kitawa (9) supported a role for the N-terminal ending by reporting that the loss of the first amino acid (Phe) greatly decreased the bioactive capacity of motilin. The results we obtained here after removal of the initial amino acid of the sequence confirmed the data of Kitawa et al. However, the motion that the N-terminal portion of the peptide was inactive, even though it included the 16 initial amino acids (11), could not be confirmed in our study. In our hands, motilin 1-9 was inactive but the N-terminal fragments extended beyond the ninth amino acid of the sequence, ie motilin 1-10, 1-11, 1-12, 1-15 etc, maintained almost complete biological activity. The explanation for the decreased activity of motilin fragment 1-16 found in the previous study (11) remains obscure. Results similar to ours were obtained by Belgium researchers working with their own synthetic analogues (T.L. Peeters, personnal communication).

Our study clarifies the ambiguity concerning the structure-activity of motilin and indicates that the N-terminal amino acid sequence 1-9 is an essential determinant for the contractile activity of motilin. Further studies aiming to develop synthetic peptides that

could act as motilin receptor agonist or antagonist should concentrate on this portion of the molecule. Therapeutic clinical interest to develop substances interacting with motilin receptors has been recently emphasized by the observation that motilin receptor agonists, such as the erythromycin derivatives (16), could represent most powerful gastrokinetic agents to improve gastric emptying dysfunction in man (17).

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