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GASTROINTESTINAL HORMONE RECEPTORS ON ISOLATED SMOOTH MUSCLE CELLS FROM GASTRIC ANTRUM OF THE RABBIT

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Abstract—The regulation by gastrointestinal polypeptide hormones of contraction and relaxation of functionally isolated smooth muscle cells from gastric antrum of the rabbit has been investigated. Gastrin, cholecystokinin (CCK-8) and motilin induced a rapid contraction of isolated cells: significant response occurred within a 5-sec incubation with these peptides and maximal response (40% decrease in cell length) after 30 sec. A higher sensitivity of smooth muscle cells to gastrin and CCK-8 than to motilin stimulations was demonstrated (EC_{50} = 10 pM for both gastrin and CCK-8 and EC_{50} = 1 nM for motilin). The minimal gastrin fragment required to get full contraction was the C-terminal pentapeptide amide common to gastrin and CCK. Proglumide inhibited gastrin- or CCK-8- but not motilin-induced contractions with an IC_{50} of 50 μM. Contraction induced by gastrin and motilin required normal levels of extracellular calcium, whereas that due to CCK-8 seemed to be independent of extracellular calcium. Vasoactive intestinal polypeptide (VIP) caused a relaxation of smooth muscle cells maximally contracted by carbachol or CCK-8 or gastrin (EC_{50} = 2.2 nM) with a parallel increase in intracellular cAMP content.

The gastrointestinal hormones gastrin and cholecystokinin play an important role in the regulation of gut motility. Cholecystokinin (CCK) is released as a hormone from the mucosa of the small intestine and as a neurotransmitter-neuromodulator from nerve terminals of the myenteric plexus [1, 2]. CCK octapeptide (CCK-8) has a direct effect on circular smooth muscle of the gallbladder and an indirect effect on longitudinal muscle of the ileum via the release of acetylcholine or substance P [3–5]. Moreover, neural and direct mediations may oppose each other in the lower esophagal sphincter of the cat: CCK-8 causes a neurally mediated relaxation whereas a direct contractile effect can be observed only after treatment of the tissue with tetrodotoxin [6].

Unlike muscle strips, isolated smooth muscle cells provide a homogeneous test system which has been used to clarify the direct effect of CCK as a contractile agent of these cells. CCK-8 mediates with high sensitivity the contraction of smooth muscle cells isolated from gastric antrum or fundus of various species (dog, guinea-pig, human) [7–9]. In contrast with CCK, the effect of the parent hormone gastrin on the contraction of smooth muscle cells has been poorly investigated. Gastrin and CCK receptors on smooth muscle cells are indistinguishable because of the similarity of the contractile effects of CCK-8 and gastrin (HG-17) on isolated smooth muscle cells [7] or on smooth muscle strips [10]. The other gastrointestinal peptide, motilin, isolated from hog small intestinal mucosa, induces contraction of the lower esophagal sphincter of the dog [11] and opossum [12] and of the dog stomach [13]. On isolated smooth muscle strips from rabbit duodenum, motilin causes a dose-dependent contraction and this stimulatory effect was found to be due to a direct effect on muscle cells, resulting in an increase of Ca^{2+} influx across the cell membrane [14]. Furthermore, recent studies have demonstrated that motilin elicited contraction of isolated cells from the guinea-pig stomach [15].

Contraction of the smooth muscle cells is dependent upon an increase in the intracellular concentration of free Ca^{2+}, which may result from an influx of extracellular Ca^{2+} through voltage-sensitive or voltage-insensitive channels or from a release of Ca^{2+} from intracellular storage sites. Contraction of gastric smooth muscle cells due to CCK-8 results in an increase in cytosolic Ca^{2+} and this effect is independent on extracellular free Ca^{2+} [16]. In contrast, removing extracellular Ca^{2+}, partially inhibited contractions induced by maximal or submaximal concentrations of pentagastrin but did not affect contractions induced by supramaximal doses of this peptide [17].

Relaxation of gastric smooth muscle cells is also under neurohormonal control. Three distinct types of receptors mediating relaxation have been identified on isolated cells: receptors for β-adrenergic agonists [18], receptors for ATP and related purine nucleotides and nucleosides [19] and receptors for Vasoactive Intestinal Polypeptide (VIP) [20]. VIP causes a dose-dependent relaxation of smooth muscle cells isolated from human or guinea-pig antrum contracted with CCK-8 [9, 20]. Moreover, high affinity binding sites for VIP have been identified on these cells [21].

In the present study we prepared smooth muscle cells from the gastric antrum of the rabbit to inves-

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tigate the characteristics of the peptidergic control of contraction and relaxation. The kinetics, stoichiometry and specificity of the contractile response induced by CCK-8, gastrin and motilin were examined together with the effect of extracellular Ca^{2+} on the contractile response of isolated cells. The role of proglumide, a competitive inhibitor of CCK-8-induced amylase release from dispersed acini and of gastrin-induced acid secretion from isolated gastric parietal cells, was determined. The biochemical events that mediate the relaxant effect of VIP were also investigated.

MATERIALS AND METHODS

Collagenase from *Clostridium histolyticum* and pronase were from Boehringer–Mannheim (F.R.G.) Gastrin (Nle^{11}-HG-13) was a gift of Professor L. Moroder (Max Planck Institut, Munich, F.R.G.). Pentagastrin (Boc-β-Ala-HG-4), motilin, VIP, soybean trypsin inhibitor (STI) and bovine serum albumin (BSA) fraction V were from Sigma Chemical Co. (St Louis, MO). Earle's balanced salt solution was from Bio-Mérieux (France). The cyclic-AMP radioimmunoassay kit was from Amersham (Bucks, U.K.). [^{125}I]VIP (2000 Ci/mmol) was from Dupont De Nemours, NEN division (France).

Medium A: 132 mM NaCl, 5.4 mM KCl, 5 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 25 mM HEPES, 0.2% glucose, 0.2% BSA, 0.02% Phenol red, pH 7.4.

Medium B: Earle's balanced salt solution containing 10 mM HEPES and 0.2% BSA, pH 7.4.

PBS: phosphate-buffered saline solution, pH 7.4.

Isolation of smooth muscle cells from gastric antrum. Smooth muscle cells from the gastric antrum of a rabbit were prepared by the method described by Bitar *et al.* [7] with minor modifications. After removing the stomach, the antral part was extensively washed with iced PBS solution. The serosal and mucosal layers were peeled off the muscular layers. Muscle tissues were minced into small pieces of 2 to 3 mm^2 and incubated in medium A containing 0.25% collagenase, 0.04% pronase, 0.01% STI and gassed with 100% O_2; after 60 min at 30°, the incubation medium was filtered through a nylon mesh. The filtrate, which contained isolated cells, was diluted with medium A and centrifuged at 150 *g* for 5 min. The cell pellet was then diluted in medium B. The remaining tissue from the first incubation was reincubated in fresh medium A for 30 min at 30° and fragments were dispersed into single cells by passages in and out the inverted wide end of a 5-ml pipette. The resulting cell suspension was filtered through a nylon mesh. Isolated cells from the two incubations were pooled and counted. Viability (estimated by Trypan blue exclusion) was always greater than 90%. This protocol usually yielded about 1×10^7 cells per rabbit antrum.

Measurement of isolated cell contraction. The cell suspension (6×10^4 cells in 0.5 ml) was added to 0.1 ml of medium B containing the agents to be tested. After 30 sec at 30°, the reaction was stopped by adding 0.05 ml of glutaraldehyde solution to a final glutaraldehyde concentration of 2% (v/v). In control experiments, 0.1 ml of medium B was used instead of the agent solution. The mean cell length was statistically determined by video-microscopic measurements (Nikon microscope with a JVC videocamera) of about 100 cells. For studies with proglumide, cells were incubated with various concentrations of the compound for 10 min. CCK-8 or gastrin (1 nM) was then added to the incubation medium and the contractile response was measured after 30 sec incubation with the peptides. The contractile response is expressed as the percentage of decrease in average cell length compared to the mean length of control cells.

Measurement of VIP-induced relaxation. Cells (6×10^4 in 0.5 ml) were preincubated with 0.1 μM VIP (time-course) or various concentrations of VIP (dose–response curve) in medium B at 30° for 5 to 60 sec. CCK-8 (1 nM), gastrin (1 nM), motilin (0.4 μM) or carbachol (0.1 μM) was then added. After a 30-sec incubation period the reaction was stopped by adding 0.1 ml of glutaraldehyde solution (final concentration 2%, v/v). In control experiments, 0.05 ml of medium B was used for preincubation instead of VIP solution. The mean cell length was determined as described above.

Binding studies. Aliquots of the cell suspension containing about 160,000 cells were incubated in small tubes with [^{125}I]VIP (0.1 nM) in the presence or not of unlabeled VIP in medium B (0.2 ml final volume) for 30 min at 30°. Bound and free fractions were separated by adding 0.6 ml of ice-cold medium B and centrifuging immediately at 8000 *g* for 1 min in a microfuge. The cell pellet was suspended in 0.1 ml of $HClO_4$ (10%) and put into vials containing 10 ml of ACS for determination of radioactivity with a liquid scintillation counter. Nonspecific binding was determined by measurements of bound radioactivity in the presence of an excess of unlabeled VIP (1 μM).

Cyclic-AMP determinations. Cyclic-AMP concentrations in isolated smooth muscle cells were measured by radioimmunoassay as follows: the cell suspension (150,000 cells/ml) was preincubated for 5 to 60 sec in medium B at 30° with 0.1 μM VIP (time course) or various concentrations of VIP (dose–response curve); then a 0.4 ml aliquot was incubated in duplicate in the presence of 10 μM IBMX for 5 min at 30°. Cells were centrifuged and resuspended in 0.4 ml of medium B. Trichloracetic acid (0.1 ml, 40%) was then added and cyclic-AMP was measured by duplicate radioimmunoassays of neutralized 0.1-ml aliquots. The amount of cyclic-AMP is expressed as picomoles per 150,000 cells.

RESULTS

Peptide-induced contractions

Kinetics. The characteristics of peptide-induced contractions are illustrated in Fig. 1. CCK-8 (1 nM), gastrin (1 nM), and motilin (0.4 μM) caused near 25% reduction in cell length within 5 sec and a maximum reduction (40%) in cell length by 30 sec. The kinetics were biphasic since contraction decreased after 30 sec to a value of about 40% of the maximal response, which persisted for at least 10 min.

Dose–response curves. To examine the dose–

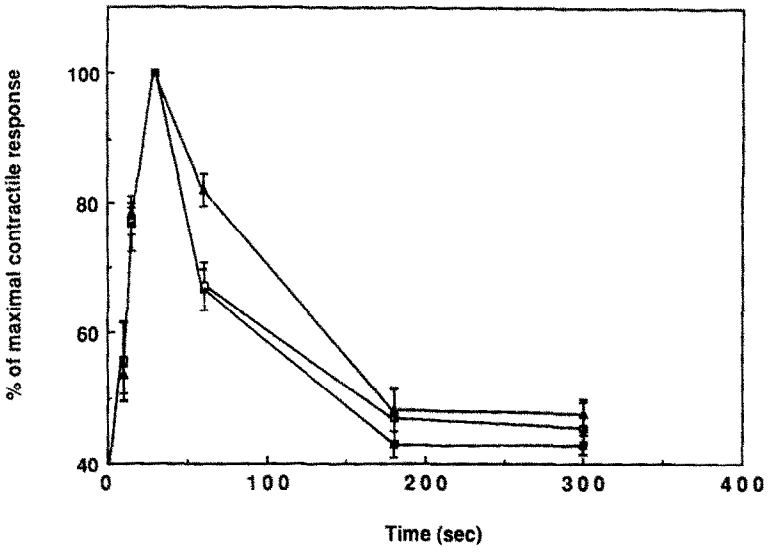

Fig. 1. Time-course of the contractile response of isolated antral smooth muscle cells in the presence of gastrin, CCK-8, and motilin. Cells (6×10^4 cells in 0.5 ml) were incubated in medium B with gastrin (1 nM), CCK-8 (1 nM), and motilin (0.4 μM) for indicated times at 30°. After fixation, the cell length of about 100 cells was determined as described in Materials and Methods. Each value is the mean ± SE of four separate experiments. Results are expressed as the percentage of maximal response (40% decrease in cell length) vs time. Gastrin (■), CCK-8 (□), motilin (▲).

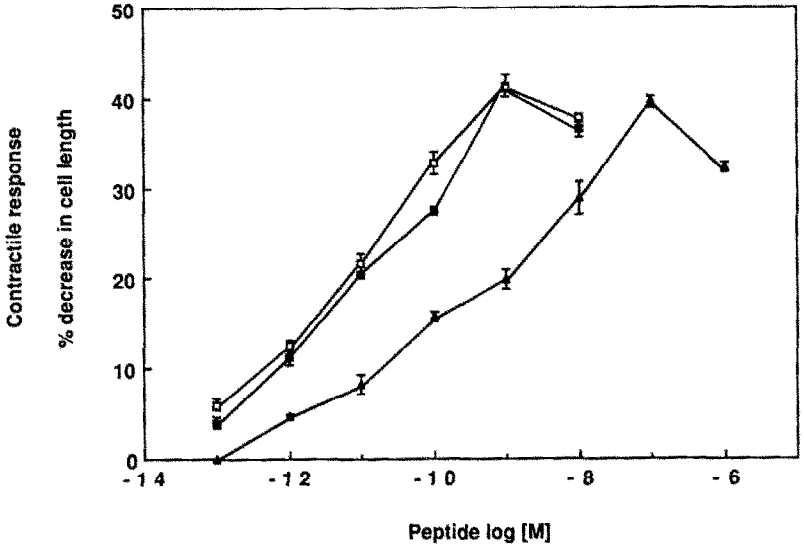

Fig. 2. Dose–response curves for gastrin-, CCK-8-, and motilin-induced contraction of isolated antral smooth muscle cells. Cells (6×10^4 cells in 0.5 ml) were incubated in medium B with indicated concentrations of peptides for 30 sec at 30°. After fixation, the cell length of about 100 cells was determined as described in Materials and Methods. Each value is the mean ± SE of four separate experiments. Gastrin (■), CCK-8, (□), motilin (▲).

response relationships of peptide-induced contractions, cells were incubated with various concentrations of peptides. Dose–response curves for CCK-8, and gastrin were superimposed: with increasing peptide concentrations, contraction increased and became maximal at 1 nM (Fig. 2). Significant contraction was induced by gastrin, and CCK-8 concentrations as low as 0.1 pM (EC_{50} values: about 10 pM). In contrast, motilin caused significant contraction at 4 pM (40 times higher than the other peptides) and the concentration required for maximal contraction (40.5 ± 2.1% decrease in cell length) was 0.4 μM (400 times higher than the concentrations of CCK-8, and gastrin) (EC_{50} = 1 nM).

Effects of various short C-terminal peptides from the gastrin/CCK family. As can be seen in Fig. 3, pentagastrin induced cell contraction in a dose-dependent fashion similar to gastrin (minimal

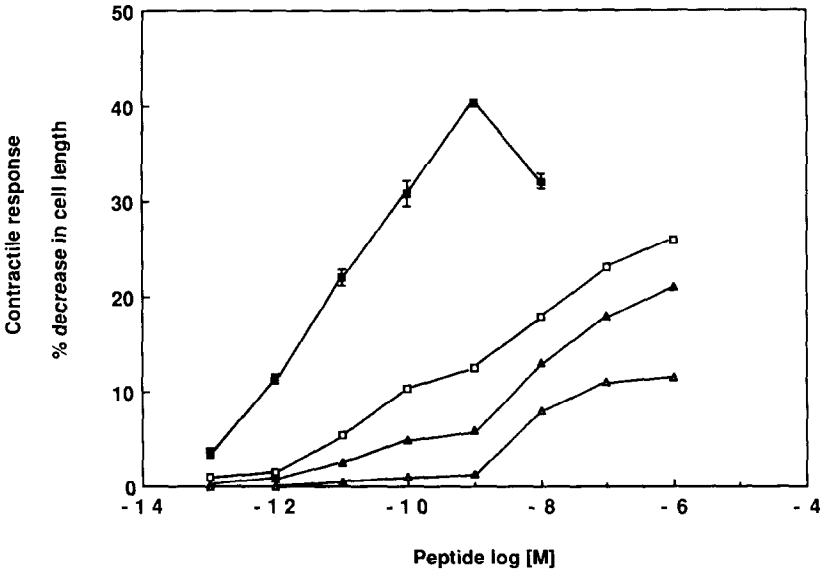

Fig. 3. Effects of C-terminal fragments from the gastrin/CCK peptide family on the contraction of isolated antral smooth muscle cells. Cells (6×10^4 cells in 0.5 ml) were incubated in medium B with indicated concentrations of peptides for 30 sec at 30°. After fixation, the cell length of about 100 cells was determined as described in Materials and Methods. Each value is the mean ± SE of four separate experiments. (■) Pentagastrin; (□) tetragastrin; (▲) Leu-Asp-PheNH$_2$; (△) Asp-PheNH$_2$.

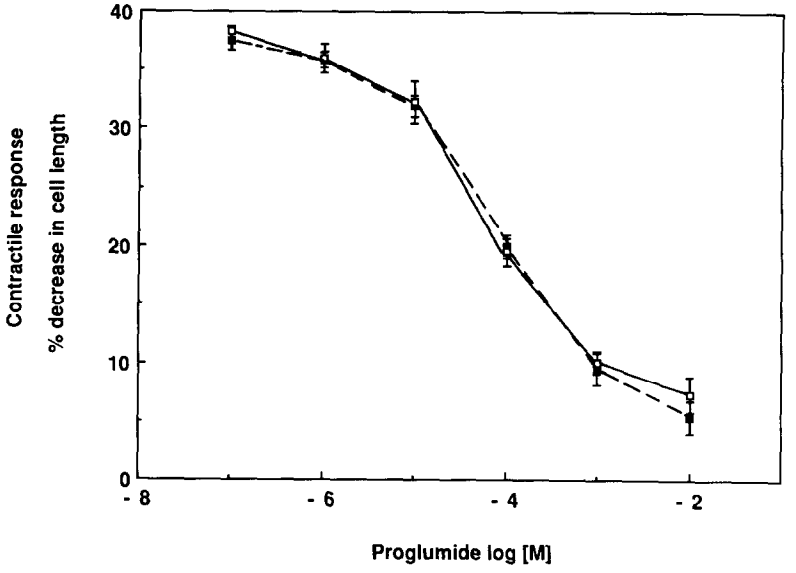

Fig. 4. Effect of proglumide on gastrin- and CCK-8-induced contraction of isolated antral smooth muscle cells. Cells (6×10^4 cells in 0.5 ml) were incubated in medium B with indicated concentrations of proglumide for 10 min at 30°. Then, gastrin (1 nM) or CCK-8 (1 nM) was added and the contractile response was measured after 30 sec incubation. After fixation, the cell length of about 100 cells was determined as described in Materials and Methods. Each value is the mean ± SE of four separate experiments. Gastrin (■), CCK-8 (□).

response at 0.1 pM, maximal at 1 nM, EC_{50} = 10 pM). The shorter peptide fragments from the C-terminal sequence (Trp-Met-Asp-PheNH$_2$, Met-Asp-PheNH$_2$, Asp-PheNH$_2$) were less potent than pentagastrin and the decrease in the response paralleled the decrease in peptide cell length.

Effect of proglumide on gastrin and CCK-8-induced contraction. The ability of proglumide to inhibit contractions induced by these peptides in isolated smooth muscle cells is shown in Fig. 4: at concentrations ranging from 1 μM to 10 mM, proglumide similarly inhibited contraction due to CCK-8 or gastrin and the concentration of proglumide causing 50% inhibition of contraction was 50 μM. As expected for a CCK/gastrin-antagonist, proglumide did not modify the response of the smooth muscle cells to motilin.

Effect of extracellular calcium on peptide-induced contraction. Incubation of cells in Ca^{2+}-depressed medium (2.5 mM EGTA) or in the presence of the

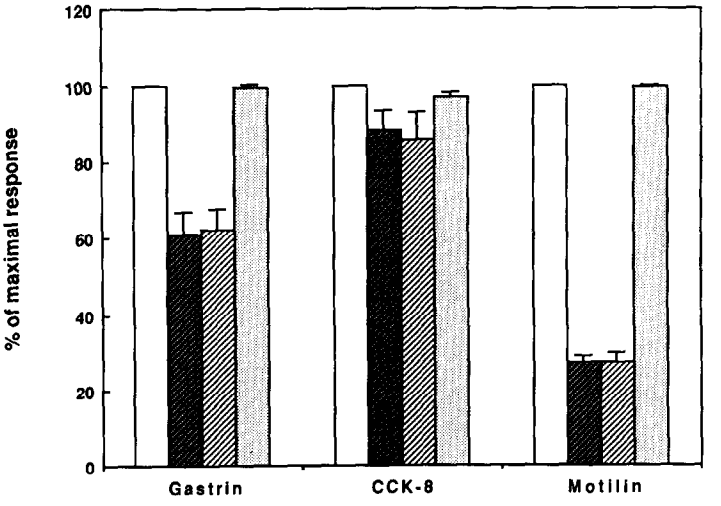

Fig. 5. Effect of extracellular calcium on the contractile response of isolated antral smooth muscle cells. Cells (6×10^4 cells in 0.5 ml) were incubated either in medium B (controls: □) or in Ca^{2+}-depressed medium (2.5 mM EGTA: ■) or in the presence of 10 μM diltiazem (▨) or in Ca^{2+}-restored (2 mM) medium (▩) for 10 min at 37°. Then, gastrin (1 nM), CCK-8 (1 nM) or motilin (0.4 μM) was added and the corresponding contractile response was determined as described in Materials and Methods. Each value is the mean ± SE of four separate experiments.

Ca^{2+}-channel blocker diltiazem (10 μM) caused significant reductions in gastrin, and motilin-induced contractions (38 ± 5% and 72 ± 3%, respectively) (Fig. 5). When normal calcium levels (2 mM) were restored, the contractile response to gastrin and motilin was fully recovered. In contrast, removing extracellular Ca^{2+} did not affect contraction caused by CCK-8. These results indicate that the effects of gastrin and motilin are dependent upon extracellular calcium, whereas the effect of CCK-8 is independent of extracellular Ca^{2+}, suggesting different intracellular mechanisms for the action of these peptides.

VIP-induced relaxation

Kinetics. The effect of VIP was measured in terms of its inhibition of contraction induced by contractile agents. The cells were preincubated with 0.1 μM VIP for 5 to 60 sec after which CCK-8, gastrin, motilin or carbachol, was added for 30 sec. The mean cell length was measured after cell fixation as described in Materials and Methods.

VIP alone (0.1 μM) did not modify the mean length of isolated smooth muscle cells. Figure 6 shows the time course of VIP-induced relaxation of cells maximally contracted by CCK-8: after 5 sec of incubation with VIP (0.1 μM), a significant relaxation was observed (28 ± 3%). Maximal relaxation occurred after 30 sec of incubation with VIP. Intracellular cyclic AMP content increased in parallel, reaching a plateau (70 pmoles cyclic AMP per 150,000 cells) by 30 sec of incubation with the peptide (Fig. 6). Similar results were obtained with gastrin, motilin or carbachol-contracted cells (data not shown).

Dose–response curves of relaxation and cyclic-AMP levels. Figure 7 shows the dose–response curve for the effect of VIP on CCK-induced contraction of cells and on intracellular cyclic AMP levels. VIP induced a dose-dependent relaxation of CCK-contracted cells and a dose-dependent increase in cyclic AMP levels with similar EC_{50}: 2.2 nM for relaxation and 2 nM for cyclic AMP. These results are in agreement with a close relationship between the relaxant effect of VIP and the increase in intracellular cyclic AMP content. Similar results were obtained with cells contracted by carbachol or gastrin (data not shown).

[^{125}I]*VIP binding.* [^{125}I]VIP can specifically bind to isolated smooth muscle cells. Specific binding was time- and temperature-dependent and reached equilibrium by 2 min at 30° (data not shown). This binding was not affected by proglumide. The IC_{50} value calculated from the inhibition of binding curves of four separate experiments was 1 ± 0.2 nM. It was closely related to the EC_{50} values found for relaxation and for the increase in cyclic AMP (2.2 nM and 2 nM, respectively).

DISCUSSION

Functionally viable isolated cells can be prepared from circular smooth muscle of rabbit gastric antrum. These cells contain muscarinic receptors that mediate contraction as previously shown [22]. GI hormones, CCK-8, gastrin, and motilin also induce contraction, suggesting the existence of specific receptors for these hormones.

Peptide-induced contraction was a rapid process occurring within 5 sec after addition of the peptide, reaching a maximum by 30 sec and then decreasing to a sustained value (about 40% of maximal contraction) whatever the stimulant used (CCK-8, gastrin, or motilin). Dose–response curves show that CCK-8 and gastrin are more potent (about 400-fold) than motilin in inducing contraction, suggesting a higher sensitivity of these cells to CCK and gastrin

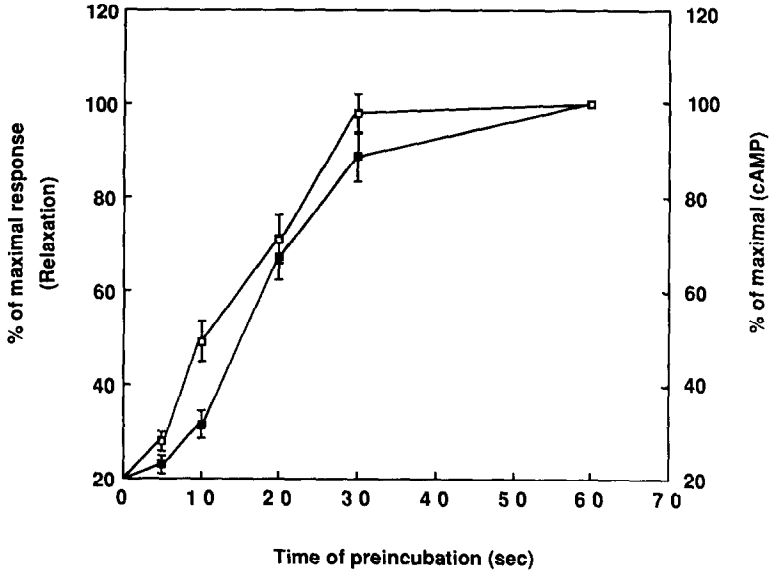

Fig. 6. Time-course for VIP-induced relaxation and cyclic-AMP accumulation in isolated antral smooth muscle cells. Cells (6×10^4 cells in 0.5 ml) were preincubated in medium B for various times with 0.1 μM VIP. Then CCK-8 (1 nM) was added for 30 sec and both the corresponding mean cell length (■) and cyclic-AMP levels (□) were measured as described in Materials and Methods. Each value is the mean ± SE of four separate experiments and results are expressed as a percentage of the maximal response.

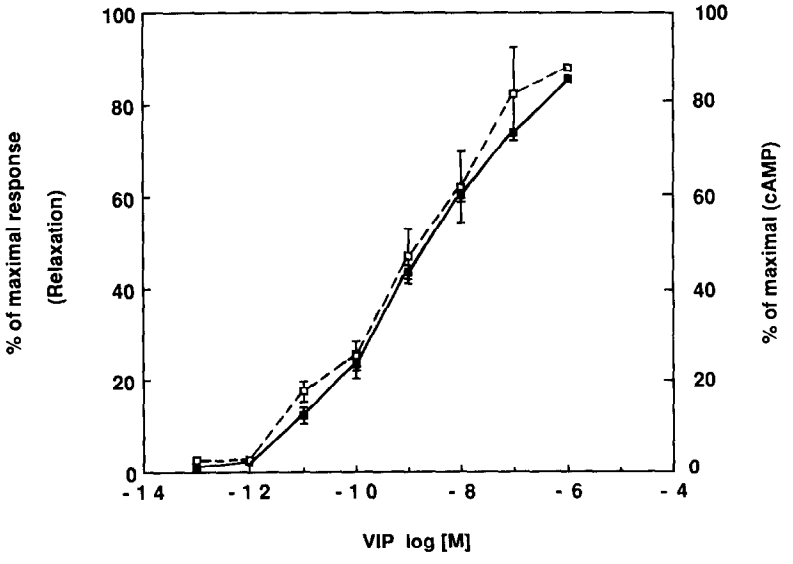

Fig. 7. Dose–response curves for VIP-induced relaxation and cyclic-AMP levels of isolated antral smooth muscle cells. Cells (6×10^4 cells in 0.5 ml) were preincubated in medium B for 1 min with the various indicated concentrations of VIP. Then CCK-8 (1 nM) was added for 30 sec and both the corresponding mean cell length (■) and cyclic-AMP levels (□) were measured as described in Materials and Methods. Each value is the mean ± SE of four separate experiments and results are expressed as a percentage of the maximal response.

than to motilin. Low doses of CCK-8 or gastrin induced contraction of rabbit cells (significant contraction occurred with 0.1 pM agonist) and the ED_{50} was about 10 pM. These data are similar to those obtained with smooth muscle cells from other species [7, 9].

CCK-8, gastrin, and pentagastrin displayed an identical profile of action: no difference can be seen in the kinetic or in dose–response data. This suggests that the minimal fragment required for full contraction is the pentapeptide amide common to gastrin and CCK. The similarity of gastrin and pentagastrin

actions on these cells contrasts with their effect on acid secretion, where pentagastrin is about 10 times less potent than gastrin [23]. With decreasing lengths of the peptide chain (C-terminal tetra-, tri- and dipeptide amide), a corresponding decrease in peptide potency was observed.

The known competitive antagonist, proglumide, was shown to inhibit both the binding and secretory activity of CCK-8 on pancreatic acini [24] and of gastrin on isolated gastric parietal cells, with the same IC_{50} value of 0.4 mM [25, 26]. In isolated smooth muscle cells from gastric antrum, proglumide also inhibited the contractile response to CCK-8 and gastrin with the same IC_{50} (about 50 μM). However, it cannot be concluded from these data that gastrin and CCK act on the same class of receptor. In order to attempt to differentiate between gastrin and CCK receptors, the requirement of extracellular calcium for the induction of full agonist activity was investigated in experiments in which cells were incubated either in Ca^{2+}-free medium containing EGTA or in the presence of the Ca^{2+}-channel blocker, diltiazem or in normal Ca^{2+} medium. The peak response to maximal doses of CCK-8 was not significantly different from the response in the presence of normal Ca^{2+} levels, whereas withdrawal of extracellular calcium caused a dramatic decrease in gastrin- and motilin-induced contractions, indicating a Ca^{2+}-dependence of the contractile response to gastrin and motilin, but not to CCK-8.

The reduction of the contractile response to gastrin and motilin in the presence of the Ca^{2+}-channel blocker diltiazem is in agreement with the presence on these these cells of "L-type" Ca^{2+}-channels involved in the hormone-induced contraction. The calcium-independence of CCK-induced contraction was previously reported by Bitar *et al.* [16]. This suggests different intracellular mechanisms for the action of these hormones.

A brief preincubation (30 sec) of smooth muscle cells with VIP inhibited the contractile response to CCK-8, gastrin, motilin or carbachol, and a parallel increase in intracellular cyclic AMP content was observed. Considering that the ED_{50} for the effect of VIP on cell relaxation or cyclic AMP levels were closely related to the IC_{50} for inhibition of [^{125}I]VIP binding, it is suggested that VIP receptors in these cells are coupled to the adenylate cyclase system and that relaxation is mediated by an increase in intracellular cyclic AMP content.

In conclusion, our results show that the gastrointestinal hormones CCK-8, gastrin, and motilin induce contraction of isolated smooth muscle cells by interacting directly through specific receptors, while VIP induces relaxation through receptor coupled to adenylate cyclase.

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