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Porcine ileal polypeptide causes an increase in cytoplasmic Ca^{2+} in both parietal and chief cells resulting in acid and pepsinogen secretion

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Porcine ileal polypeptide, an enterooxyntin isolated from distal small intestinal mucosal epithelium, has been observed to stimulate gastric acid secretion in vivo as well as in vitro (Wider, M.D. et al. (1984) Endocrinology 115, 1484–1491, Wider M.D. et al. (1986) Endocrinology 118, 1546–1550). We report here that porcine ileal polypeptide stimulates both acid (aminopyrine accumulation) and pepsinogen secretion in isolated, enriched populations of guinea pig parietal and chief cells in a dose-dependent manner. Further, 10^{-9} M porcine ileal polypeptide caused an increase in cytoplasmic Ca^{2+} concentration in both parietal and chief cells similar in magnitude to that observed with gastrin-17 (10^{-8} M) (as measured by both fura-2 and aequorin) and cholecystokinin octapeptide (CCK-OP) (10^{-8} M), respectively. Porcine ileal polypeptide has been observed to cause no stimulation of cAMP production in gastric glands from guinea pigs (Gespach, C., personal communication) nor is there any effect of medium Ca^{2+} depletion on acid production observed with guinea pig gastric mucosal sections. It is concluded that porcine ileal polypeptide, at concentrations similar to circulating levels observed in plasma of normal pigs ($5 \cdot 10^{-9}$ M), acts directly on the parietal and chief cells to cause the mobilization of intracellular Ca^{2+} from the stores resulting in acid and pepsinogen secretion. These experiments demonstrate that this peptide is a potent enterooxyntin and chief cell secretagogue which acts via the same signal transduction mechanisms as gastrin and cholecystokinin.

Introduction

The regulation of parietal and chief cell function by histamine, muscarinic cholinergic agents and gastrin has been extensively studied and the subcellular signal transduction mechanisms characterized. While these primary regulators of gastric function are well defined, the hormonal stimulus from the distal small intestine [1–4] which exerts a delayed effect on acid secretion [1,2] is less well understood. Portacaval shunt has been observed to result in hypersecretion of gastric acid and the development of duodenal ulceration in man [5] and to increase basal but not pentagastrin-stimulated acid secretion [6] in animals. Marked proliferation of gastric ECL cells has also been identified in rats with portacaval shunts [6]. These effects have been attributed to a hormonal factor from the small intestine that is rapidly degraded

Abbreviations: fura-2, 1-(2-(5′-carboxyoxazol-2′yl)-6-aminobenzofuran-5-oxy)-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; CCK-OP, cholecystokinin octapeptide.

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by the liver [5,7]. We report here the investigation of the effect of porcine ileal polypeptide, an enterooxyntin isolated from the ileal mucosa of pigs [8], on acid and pepsinogen secretion and intracellular Ca^{2+} mobilization using isolated, enriched populations of parietal and chief cells, respectively. Porcine ileal polypeptide has been previously reported to cause gastric acid secretion in vivo and in vitro and has been identified in plasma from normal adult pigs [9]. Further, recent study has demonstrated a marked effect on cell growth in cultured, mixed cell populations from gastric and intestinal epithelium (Moyer, M.P., personal communication). The studies reported here demonstrate that porcine ileal polypeptide in concentrations similar to the levels observed in plasma causes increased acid and pepsinogen secretion by stimulating the release of Ca^{2+} from the intracellular stores.

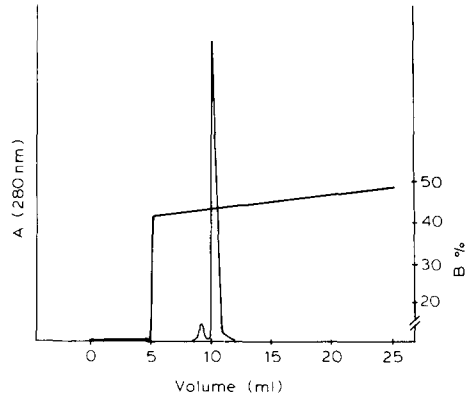

Fig. 1. Final purification of porcine ileal polypeptide by reverse phase $C_{1/8}$ HPLC. Porcine ileal polypeptide purified as described previously [8] was loaded onto a $C_{1/8}$ reverse phase column (Pharmacia Pro RPC) eluted with a 42 to 47% gradient (A: 0.1% trifluoroacetic acid (TFA) in H_2O, B: 0.1% TFA in acetonitrile) at 0.5 ml/min. The 214 nm/280 nm signal ratio indicated that the small 280 nm peak eluting just ahead of the porcine ileal polypeptide peak represented less than 0.01% of the total mass. The major peak was lyophilized, and stored at −20 °C in sealed vials.

Materials and Methods

Agents. All chemicals used were reagent grade unless otherwise stated and were from Sigma (U.S.A.). Collagenase type I (150 U/mg *Clostridium histolyticum* was from Sigma (U.S.A.). Aequorin was purchased from Dr. Blinks (Mayo Foundation, U.S.A.), and fura-2 acetoxymethyl ester was obtained from Dougindo (Japan). Percoll was from Pharmacia (Sweden). Human [Leu^{15}]-gastrin 17 from Sigma (U.S.A.) was kept at −20 °C in a stock solution (10 μM) in 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl, 10% dimethylsulfoxide, 675 U/ml of Kallikrien inhibitor Trasylol from Boehringer-Mannheim (F.R.G.). Amino[^{14}C]pyrine dimethyl amine (110 mCi/mmol) was from New England Nuclear (U.S.A) and fetal calf serum was from Gibco (U.S.A.). Porcine ileal polypeptide used for this study was prepared as described previously [8] except that fractionation on a reverse phase C_8 column was employed for final purification (Fig. 1).

Isolation of parietal and chief cells. Dispersed mucosal cells from guinea pig gastric mucosa (Hartley, male, 250 g) were prepared by digestion with collagenase (0.01%) and dipase (0.08%) in tissue culture medium (RPMI 1640) containing 10% fetal calf serum and by brief mechanical disruption with EDTA (0.5 mM) in Ca^{2+}-Mg^{2+} free Krebs-Ringer bicarbonate buffer by a previously described [10,11] modification of the method of Berglindh [12]. Enriched cell populations were prepared by Percoll density gradient ultracentrifugation (30000 × *g* for 15 min at 4 °C). Parietal or chief cell-enriched fractions were obtained from the interface (density 1.043–1.050 g/ml) and from the bottom (density 1.062–1.076 g/ml) of the gradient, respectively. The pepsinogen content of the chief cell enriched fraction was 8-times that of the parietal cell fraction [21]. Cell viability was determined by exclusion of 0.4% Trypan blue. Almost 95% of the cells were viable. Cell suspensions were diluted with excess medium 199 (tissue culture medium which had been modified by Earles medium) and recentrifuged (350 × *g* for 10 min at 4 °C) to remove Percoll from the cell suspension. The resultant pellet (10^7 parietal or chief cells) was resuspended in medium 199.

Cytoplasmic free Ca^{2+} determination. $[Ca^{2+}]_i$ was determined by the photoprotein aequorin [13] and fluorescent probe fura-2 (acetoxymethyl ester) methods [14]. The index of acid secretion was measured by amino[^{14}C]pyrine accumulation [12] in parietal cells.

Pepsinogen assay. Pepsinogen activity was determined by the method of Anson and Mirsky [15] which entails adding 100 μl of sample supernatant or 20-fold diluted, sonicated pellet to 400 μl of acid solution (320 μl H_2O + 80 μl 0.3 M HCl, pH 1.8) containing 2.5% human hemoglobin followed by incubation at 37°C for 10 min. The reaction was stopped by the addition of 1 ml of 5% trichloroacetic acid followed by centrifugation at 750 × *g* for 10 min. 500 μl of supernatant was added to 2.5 ml of 0.5 M Na_2CO_3 + 250 μl of 0.1 M phenol reagent, incubated for 15 min at 24°C and the absorbance read at 640 nm. Tyrosine was used as a standard and appropriate blanks with trichloroacetic acid were run with each assay. The effect of various concentrations of porcine ileal polypeptide was examined to establish a dose response for the peptide on acid and pepsinogen secretion in the enriched parietal and chief cell fraction, respectively. The optimal concentration of porcine ileal polypeptide (10^{-9} M) observed in these experiments was subsequently used to determine the Ca^{2+} response as indicated by photoprotein aequorin and fura-2 assay.

Results

Porcine ileal polypeptide was observed to cause a potent stimulation of aminopyrine accumulation as an index of acid secretion in a dose-dependent manner (Fig. 2). The maximal response of the parietal cells was observed at 10^{-8} M for porcine ileal polypeptide resulting in an amino[^{14}C]pyrine ratio increase from 10.50 ± 0.23 to 25.31 ± 5.31. The maximal response was not quite as great as that obtained with gastrin 10^{-7} M (32.91 ± 2.32) but was observed at an 1 log unit lower concentration (10^{-8} M porcine ileal polypeptide).

Porcine ileal polypeptide also caused a very potent stimulation of pepsinogen release in a dose-dependent manner (Fig. 2) with a maximal response (percentage of total pepsinogen; 3.66 ± 0.81 to 8.19 ± 0.05 at 10^{-8} M) observed at 10^{-9}–10^{-7} M. The maximal response to porcine ileal polypeptide by the chief cell was almost similar to that observed with CCK-OP which gave an equivalent maximal response at 10^{-8}–10^{-7} M (7.45 ± 0.15 and 8.59 ± 0.50, respectively). Gastrin (10^{-7} M) and CCK-OP (10^{-7} M) each failed to cause pepsinogen release and aminopyrine accumulation, respectively, without affecting $[Ca^{2+}]_i$ change.

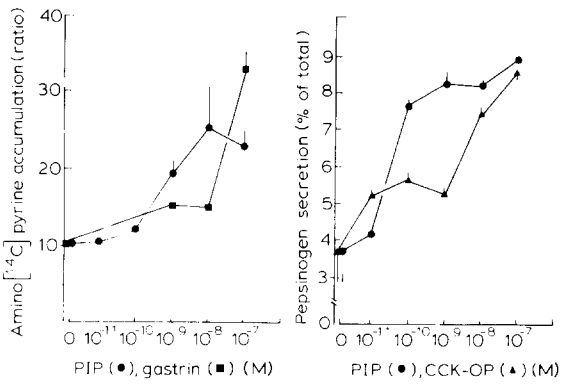

Fig. 2. Porcine ileal polypeptide (PIP)-induced amino[^{14}C]pyrine accumulation and pepsinogen secretion. The accumulation of amino[^{14}C]pyrine as an index of acid secretion was determined by the method of Berglindh [12] and the results are expressed as a ratio using the previously described formula [12]. Parietal cells (10^6/ml) were incubated in a final volume of 1 ml in Hanks' balanced salt solution containing 0.5 mM dithiothreitol with constant gassing 95% O_2/5% CO_2. The cells were incubated with the indicated agents for 15 min and incubation was terminated by centrifugation at 12000 × *g* for 30 s. Data are presented as mean ± S.E. from three separate experiments. Chief cells (10^6) were incubated for 30 min at 37°C in medium 199 in a final volume of 600 μl with constant gassing 95% O_2/5% CO_2. Incubation was terminated by centrifugation at 12000 × *g* for 30 s and both pellet and supernatant assayed for pepsinogen content by the method of Anson and Mirsky [15] as described in Materials and Methods. Pepsinogen release was calculated as a percentage of total pepsinogen activity present in the medium in relation to that in the cell pellet. The data are presented as mean ± S.E. from three separate experiments.

Aequorin luminescence indicated an apparent $[Ca^{2+}]_i$ of 4.0 μM or 7.0 μM in parietal and chief cells, respectively, (Fig. 3) which was at least 1 log unit higher than that measured by fura-2 (see, Fig. 4). The reason for this high value is not clear and the precise location of aequorin in parietal and chief cells introduced by dimethyl sulfoxide is not certain. However, it does not necessarily follow that the high $[Ca^{2+}]_i$ observed in the aequorin-loaded cells is due to increased permeability in light of the fact that even in the absence of medium Ca^{2+} and the presence of 2 mM EGTA the resting $[Ca^{2+}]_i$ indicated by aequorin luminescence is 3.5 μM for parietal and 4.5 μM for chief

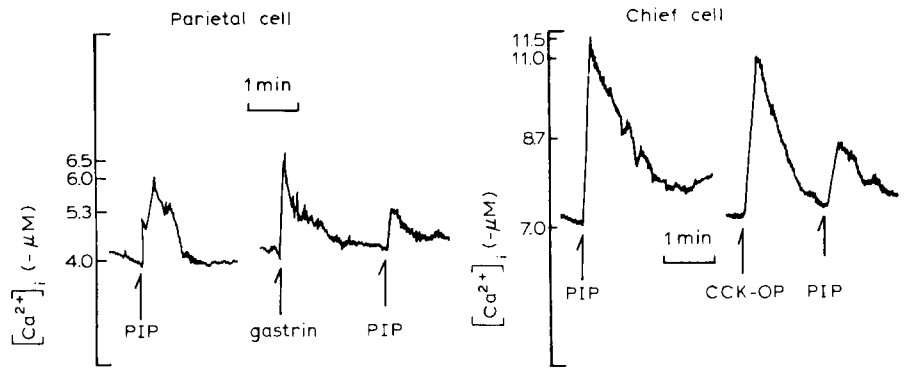

Fig. 3. Cytosolic free Ca^{2+} concentration $[Ca^{2+}]_i$ in response to secretagogues as measured by aequorin luminescence. Isolated parietal or chief cells were incubated ($5 \cdot 10^6$ cells/150 μl) at 24° C for 6 min in Hepes-Tyrode's solution (150 mM NaCl + 5 mM KCl + 0.2 mM KH_2PO_4 + 0.8 mM K_2HPO_4 + 1 mM $MgSO_4$ + 5 mM glucose + 10 mM sodium Hepes with 1 mM ATP and 2 mM EGTA) following the addition of 10 μl of aequorin (0.3 mg/ml aequorin in 5 mM Hepes + 50 mM KCl + 7 mM EGTA) as previously described [18]. Dimethyl sulfoxide was added stepwise during the incubation to a final concentration of 6% [23]. Incubation was terminated by the addition of 900 μl Hepes-Tyrode's solution followed by centrifugation at 12000 × *g* for 15 s. The cells were washed once and the resultant cell pellet resuspended in 10 ml of Hepes-Tyrode's solution. $CaCl_2$ was added to 1 ml of the cell suspension ($5 \cdot 10^5$ cells/ml) to a final concentration of 1 mM in a cuvette and the appropriate test agent added after the signal of Ca^{2+}-aequorin complex outside the cells disappeared. Aequorin luminescence was determined with a Platelet Ionized Calcium Aggregometer (PICA) (Chrono Log, U.S.A.) with constant stirring at 37° C. $[Ca^{2+}]_i$ was calculated as described [18]. Porcine ileal polypeptide was used at 10^{-9} M from a stock solution of 1 mg/ml in 10^{-5} M HCl. Gastrin and CCK-OP were used at 10^{-8} M from stock solutions of 10^{-5} M peptide in 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl + 10% dimethyl sulfoxide + 675 U/ml Trasylol + 0.2% bovine serum albumin. The gastrin used for these experiments was human gastrin 17 substituted with leucine at position 15. The trace is a representative of at least three similar experiments.

cells and that a transient increase in $[Ca^{2+}]_i$ is detectable upon stimulation of these parietal and chief cells with gastrin, CCK-OP and porcine ileal polypeptide. In addition, the resting level of $[Ca^{2+}]_i$ of the aequorin-containing parietal or chief cells monitored by fura-2 was not significantly different from that of the aequorin-free cells (see, Fig. 4 legend).

The aequorin signal is dominated by the highest $[Ca^{2+}]_i$ and has been interpreted as reflecting local elevation of Ca^{2+} [16].

Porcine ileal polypeptide (10^{-9} M) caused a rise in $[Ca^{2+}]_i$ of 2.0 μM and 4.5 μM in parietal and chief cells, respectively, which was similar in magnitude to that observed with gastrin (10^{-8} M, parietal cell, 2.5 μM final rise) and CCK-OP (10^{-8} M, chief cell, 4.0 μM final rise).

To determine whether porcine ileal polypeptide and gastrin (or CCK-OP) share common Ca^{2+} store(s), parietal (or chief) cells were prestimulated with gastrin (or CCK-OP) and porcine ileal polypeptide added after $[Ca^{2+}]_i$ had reached a steady-state level. Prestimulation of parietal or chief cells with a maximal dose of gastrin or CCK-OP, respectively, substantially reduced the response to porcine ileal polypeptide in both parietal (45% decrease) and chief cells (62.3% decrease), suggesting that porcine ileal polypeptide acts on the same Ca^{2+} pathway(s) as gastrin and cholecystokinin. This is identical with the facts that the Ca^{2+} pool is deleted by the first stimulation and that this pool is replenished by the Ca^{2+} entered from outside the cells in a sustained phase [17]. This effect was not due to the consumption of aequorin in response to the initial stimulation since the addition of 1 μM Ca^{2+}-ionophore ionomycin was still capable of eliciting an increase in $[Ca^{2+}]_i$ following prestimulation that was equal to the response to ionomycin observed without prestimulation (12.0 μM final rise following gastrin prestimulation, 11.5 μM final rise by ionomycin alone in parietal cells).

The $[Ca^{2+}]_i$ response to porcine ileal polypeptide monitored with fura-2 also demonstrated a stimulation of Ca^{2+} mobilization from intracellular stores. The resting $[Ca^{2+}]_i$ in parietal and

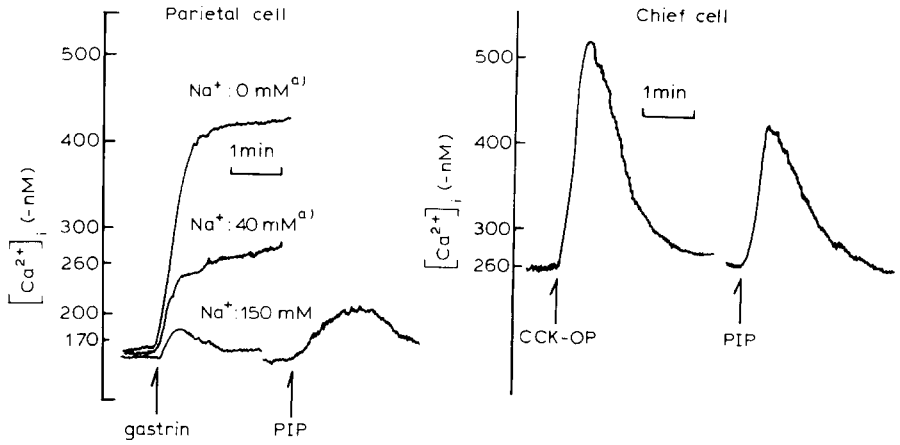

Fig. 4. Cytosolic free Ca^{2+} response to secretagogues as indicated by fura-2 fluorescence. Cells were loaded with fura-2 by incubation of parietal or chief cells (10^7/10 ml) with 2 μM fura-2 (acetoxymethyl ester) in medium 199 containing 10 mM Hepes and 25 mM $NaHCO_3$ (pH 7.4) with constant gassing 95% O_2/5% CO_2 for 5 min at 37 °C followed by a 2-fold dilution with the same medium and continued incubation for another 10 min. The cell suspension was then twice washed and centrifuged twice at 100 × g for 5 min and then resuspended in 20 ml of Hepes-Tyrode's solution. Test agents and $CaCl_2$ were added to 10^6 cells in 2 ml in a cuvette to a final concentration of Ca^{2+} of 1 mM. The ionic strength was adjusted with choline chloride ([a)]) and the fura-2 fluorescence read on a CAF 100 spectrophotometer (Japan Spectroscopic Company, Ltd. Japan). Emission wavelength was 500 nm while the excitation wavelength was either 340 nm/380 nm ratio or was maintained at 380 nm or 340 nm for the entire measurement period. $[Ca^{2+}]_i$ values are calculated from fura-2 ratios (R) by the equation: $[Ca^{2+}]_i = K(R - R_{min})/(R_{max} - R)$ where R_{min} and R_{max} are 340 nm/380 nm ratios obtained in zero (final concentration; 4 mM EGTA + 0.1% Triton X-100) or saturating (final concentration; 5 mM $CaCl_2$) Ca^{2+} concentrations. K is the product $K_d(F_o/F_s)$, where K_d is the effective dissociation constant (224 nM), F_o is the 380 nm excitation efficacy in the absence of Ca^{2+} and F_s is the 380 nm excitation at saturating Ca^{2+} concentrations. Porcine ileal polypeptide (PIP) was tested at 10^{-9} M while CCK-OP and gastrin were used at 10^{-8} M from the stock solutions described for Fig. 3. The trace is a representative of at least three similar experiments. If fura-2 (2 μM) was loaded with the aequorin-containing cells (see, Fig. 3 legend), the resting level of $[Ca^{2+}]_i$ of the parietal or chief cell was 206.0 ± 15.4 nM (n = 3) and 296.0 ± 32.0 nM (n = 3), respectively. The resting level of $[Ca^{2+}]_i$ of the aequorin-free parietal or chief cell monitored by fura-2 was 169.5 ± 23.7 nM (n = 6) and 260.2 ± 27.8 nM (n = 6), respectively.

chief cells was 169.5 ± 23.7 nM and 260.2 ± 27.8 nM, respectively (Fig. 4). Fura-2 fluorescence was evaluated by a 340/380 nm ratio as previously described [14]. The resting $[Ca^{2+}]_i$ of the parietal cells was lower than that observed in chief cells which contain abundant secretory granules extending into the apical region. Gastrin induced a much smaller maximal increase in $[Ca^{2+}]_i$ (35.3 nM final rise) in parietal cells as indicated by fura-2 when compared with the results obtained with aequorin in the presence of 150 mM Na^+ (physiologic saline). Previous studies [17] have indicated that gastrin stimulation of Ca^{2+} release into the cytoplasm is from the apical portion of the parietal cell and may be removed by a Na^+/Ca^{2+} exchanger, calmodulin-regulated Ca^{2+} pump or both. This interpretation is supported by the fact that depletion or elimination of Na^+ from the incubation medium caused a significant increase in the $[Ca^{2+}]_i$ response to gastrin as measured by fura-2. This effect is also observed with quin-2 [11]. Porcine ileal polypeptide (10^{-9} M) and gastrin (10^{-8} M) each caused a similar slow upstroke to a peak (35–40 nM final rise) followed by a decline in the fura-2 signal, suggesting that porcine ileal polypeptide exerts it's effects on the parietal cell via the same Ca^{2+} pathways(s) as gastrin.

Intracellular Ca^{2+} mobilization in chief cells in response to porcine ileal polypeptide (10^{-9} M) and CCK-OP (10^{-8} M) was similar. The fura-2 signal demonstrated a rapid rise in $[Ca^{2+}]_i$ (porcine ileal polypeptide 160 nM and CCK-OP 289 nM final rise) followed by a decline to baseline. The chief cell response is much more rapid than that observed in the parietal cell and the

similarity of response to porcine ileal polypeptide and CCK-OP indicates that they act through a common Ca^{2+} mobilization pathway.

Discussion

The results of these and previous experiments demonstrate that porcine ileal polypeptide is a potent enterooxyntin of physiologic significance. This peptide has been identified and chemically characterized in plasma from normal adult pigs [9] and has been localized to superficial epithelial cells in the distal ileum by immuno-histochemical methods [22]. The fact that the HPLC purified preparation of porcine ileal polypeptide used for these experiments was even more potent than gastrin and CCK-OP rules out the possibility that the cellular response was due to a contaminant. Further, the response observed in these experiments was elicited by a concentration of porcine ileal polypeptide (10^{-9} M) which was quite close to that observed in the blood of normal, adult pigs ($5 \cdot 10^{-9}$ M).

The identification of the signal transduction mechanism for this peptide indicates that it acts through the same pathway(s) as gastrin in parietal cells and CCK-OP in chief cells. It is unlikely that porcine ileal polypeptide is acting on receptors specific for gastrin (dissociation constant (K_d) = $4.098 \cdot 10^{-9}$ M, receptor number per parietal cell = 79000) and for CCK-OP ($K_d = 1.604 \cdot 10^{-10}$ M, receptor number per chief cell = 17500 (high affinity) and $K_d = 1.088 \cdot 10^{-8}$ M, receptor number = 281000 (low affinity)) [21] since it is more potent than either of these agents. It is, of course, possible that porcine ileal polypeptide contains a sequence similar to CCK-OP, however, one would expect a somewhat lower rather than a higher activity if that was the case. Porcine ileal polypeptide did not affect the binding of ^{125}I-gastrin to its receptor of the parietal cell (not shown). We have identified over 40 residues in the primary sequence and have observed no homology to any known protein sequence.

The signal transduction mechanisms for gastrin and CCK-OP acting on parietal and chief cells have been extensively defined. Gastrin has been observed to cause the release of Ca^{2+} from the smooth-surfaced membranes of the parietal cell that comprise the tubulovesicular elements and canalicular membranes [17,19] and CCK-OP has been shown to cause Ca^{2+} release from the endoplasmic reticulum [20,21] in chief cells. The Ca^{2+} response in both parietal and chief cells is mediated by inositol 1,4,5-triphosphate (IP_3) acting on the microfilaments [11,19,21].

The original experiments which were run using guinea pig gastric mucosal sections [8] were not affected by the addition of EGTA to the medium and porcine ileal polypeptide caused no stimulation of cAMP in isolated guinea pig gastric glands (Gespach, C., personal communication). Hence, we conclude that porcine ileal polypeptide acts to liberate Ca^{2+} from the intracellular stores by a pathway independent of adenylate cyclase, most probably via an IP_3 transduced signal. Experiments are in progress to study the effect of Ca^{2+} ionophores and IP_3 on the response to porcine ileal polypeptide by parietal and chief cells.

Porcine ileal polypeptide is an important physiologic regulator of gastric function. It has been observed to stimulate gastric acid and pepsinogen secretion in a dose dependent manner at concentrations equal to those found in plasma and also causes proliferation of gastric and intestinal epithelial cells in culture (Moyer, M.P., personal communication).

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