Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8968–8971, November 1989
Medical Sciences

Experimental pancreatitis is mediated by low-affinity cholecystokinin receptors that inhibit digestive enzyme secretion

(lysosomes/cathepsin B/exocrine secretion)

A. K. SALUJA, M. SALUJA, H. PRINTZ, A. ZAVERTNIK, A. SENGUPTA, AND M. L. STEER*

Department of Surgery, Beth Israel Hospital and Harvard Medical School, Harvard Digestive Diseases Center and Charles A. Dana Research Laboratories, Boston, MA 02215

Communicated by Eugene Braunwald, August 16, 1989 (received for review April 25, 1989)

ABSTRACT **Rats infused with a supramaximally stimulating dose of the cholecystokinin (CCK) analog caerulein develop acute edematous pancreatitis. Using CCK-JMV-180, a recently developed CCK analog that acts as an agonist at high-affinity CCK receptors but antagonizes the effect of CCK at low-affinity receptors, we have determined that caerulein induces pancreatitis by interacting with low-affinity CCK receptors. Those low-affinity receptors mediate CCK-induced inhibition of digestive enzyme secretion from the pancreas. Our observations, therefore, suggest that this form of experimental pancreatitis results from the inhibition of pancreatic digestive enzyme secretion.**

Rats infused with supramaximally stimulating concentrations of cholecystokinin (CCK) or of its decapeptide analog caerulein rapidly develop a form of acute experimental pancreatitis that is characterized by hyperamylasemia, pancreatic edema, and acinar cell vacuolization (1–3). Using nonselective CCK receptor antagonists, previous studies have demonstrated that caerulein induces pancreatitis by interacting with CCK receptors (4–6). The pancreatic acinar cell is believed to possess two classes of CCK receptors—one with higher affinity and the other with lower affinity for CCK (7–8). Studies characterizing acinar cell secretion in the presence of increasing CCK concentrations typically reveal a biphasic dose–response relationship with stimulation at low CCK concentrations and inhibition at supramaximally stimulating concentrations. Recently, a synthetic CCK analog, CCK-JMV-180, has been developed that does not cause inhibition of acinar cell secretion when supramaximally stimulating concentrations are used (9). Based on recently reported binding studies, CCK-JMV-180 is believed to interact with both classes of pancreatic CCK receptors but to act as an agonist only at the higher-affinity class. Furthermore, CCK-JMV-180 is believed to act as an antagonist at the lower-affinity CCK receptors, which appear to mediate high dose inhibition of pancreatic enzyme secretion (10, 11).

We reasoned that studies evaluating the ability of CCK-JMV-180 to induce pancreatitis should define unambiguously whether caerulein induces pancreatitis via interactions with the higher- or lower-affinity class of CCK receptors. For example, if caerulein induces pancreatitis by interacting with the higher-affinity receptor class, CCK-JMV-180 should also induce pancreatitis. On the other hand, if caerulein induces pancreatitis by interacting with the lower-affinity class of CCK receptors, CCK-JMV-180 (*i*) should not induce pancreatitis and (*ii*) should prevent caerulein-induced pancreatitis. Studies evaluating this issue are reported in this communication.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Male Wistar rats, weighing ≈75 g were obtained from Charles River Laboratories. CCK-JMV-180, an analog of the C-terminal heptapeptide of CCK with the structure of Boc-Tyr(SO_3)-Nle-Gly-Trp-Nle-Asp-(2-phenyl-ester) (Boc-, butoxycarbonyl-; Nle, norleucine) (9), was purchased from Research Plus (Bayonne, NJ). Caerulein was obtained from Peninsula Laboratories; collagenase and soybean trypsin inhibitor were from Worthington Biochemicals. All other reagents were of the highest purity commerically available.

***In Vitro* Amylase Secretion.** Dispersed rat pancreatic acini were prepared by collagenase digestion and gentle shearing as described (12). Freshly prepared acini were preincubated in 10 ml of Hepes/Ringer's buffer (pH 7.4) containing 118 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.01% soybean trypsin inhibitor, and Eagle's basal amino acids. The buffer was saturated by bubbling with O_2. After a 20-min preincubation, the acini were washed twice with the buffer and resuspended in the same buffer containing various concentrations of secretagogues. Amylase secretion at 37°C was measured over the ensuing 30 min (expressed as a percent of total amylase content of the acini). Net stimulated secretion of amylase was calculated by subtracting the secretion in the absence of secretagogue (i.e., "basal" secretion) from the secretion of amylase noted in the presence of secretagogue.

***In Vivo* Amylase Secretion.** Rats were anesthetized with an intraperitoneal injection of pentobarbital (40 mg/kg) and a PE-50 catheter was placed in the jugular vein as described (1). A midline laparotomy was performed and a PE-10 cannula was introduced into the common biliopancreatic duct for a distance of ≈0.5 cm, after extraduodenal incision of the duct. The cannula was brought out through a stab wound and the laparotomy incision was closed. Anesthesia was maintained, as needed, by periodic intraperitoneal administration of pentobarbital, and body temperature was supported with a heating pad placed under the animals. After an initial 30-min period of stabilization, pancreatic secretions were collected at 30-min intervals for 3 hr. During this time, animals were infused with saline alone or with saline containing the various concentrations of secretagogues at a rate of 0.42 ml/hr.

Experimental Pancreatitis. Rats were anesthetized and a cannula was placed in the jugular vein as described above. They were housed individually in shoe-box cages and allowed to recover overnight from anesthesia. Patency of the cannula was maintained by infusion of 0.9% saline containing heparin (20 units/ml) at a rate of 0.42 ml/hr. Conscious rats were then infused at the same rate with either heparin/saline solution alone or with heparin/saline solution containing caerulein (5

Abbreviations: CCK, cholecystokinin; CDE, choline deficient ethionine supplemented.

*To whom reprint requests should be addressed at: Department of Surgery, Beth Israel Hospital, 330 Brookline Avenue, Boston, MA 02215.

$\mu g \cdot kg^{-1} \cdot hr^{-1}$), CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$), or a combination of caerulein and CCK-JMV-180 (5 μg and 5 $mg \cdot kg^{-1} \cdot hr^{-1}$, respectively). In other experiments, animals were infused with a lower dose of caerulein (0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) alone or with somatostatin (4 $\mu g \cdot kg^{-1} \cdot hr^{-1}$). After infusion with these agents for 3.5 hr, the animals were sacrificed by cervical dislocation and blood was collected for serum amylase determination. Fragments of pancreas were prepared for light microscopic examination and for quantitation of pancreatic edema as described below. Subcellular fractionation of homogenized pancreas samples was performed as described (13).

Assays. α-Amylase activity was measured with soluble starch as the substrate according to the method of Bernfeld (14). Cathepsin B was measured as described (13). Pancreatic edema was quantitated by measuring the weight of a pancreas fragment immediately after harvesting (wet weight) and after desiccation for 36 hr at 150°C (dry weight). The results were calculated as (wet weight − dry weight)/wet weight and are expressed as a percent.

Microscopy. Rat pancreatic tissue samples were fixed in a mixture of 95% ethanol/saturated picric acid/formalin/acetic acid (80:15:5, vol/vol/vol). After paraffin embedding and sectioning, tissues were stained with hematoxylin and eosin and examined by a "blinded" observer.

Data Presentation. The data reported in the communication represent the mean ± SEM values for replicate determinations obtained with at least three animals in each group. Bars in the figures represent SEM values and their absence indicates that the SEM was smaller than the size of the symbols. The statistical significance of differences was evaluated by *t* test for unpaired observations and analysis of variance, and significant differences were defined as those associated with a P value of <0.05.

RESULTS

***In Vitro* Amylase Secretion.** The effects of caerulein and CCK-JMV-180 on *in vitro* amylase secretion from rat pancreatic acini are shown in Fig. 1. As has been previously noted by many investigators, caerulein causes dose-dependent stimulation and, at higher concentrations, inhibition of amylase secretion. The maximal rate of amylase secretion was observed in the presence of 0.1–1.0 nM caerulein, while concentrations of caerulein >1 nM inhibited secretion. In contrast to caerulein, CCK-JMV-180 was noted to cause only dose-dependent stimulation of amylase secretion. The maximal rate of amylase secretion was observed in the presence of ≈1 μM CCK-JMV-180, while higher concentrations neither further increased nor decreased this rate of secretion. Studies performed in the presence of both caerulein and CCK-JMV-180 indicated that 1 μM CCK-JMV-180 could prevent the inhibition of amylase secretion, which was otherwise observed when the concentrations of caerulein exceeded 1 nM. Similar results have been recently reported by others (9–11). These findings indicate that both caerulein and CCK-JMV-180 are agonists at the higher-affinity receptors that mediate the stimulation of amylase secretion. On the other hand, these results indicate that caerulein and CCK-JMV-180 have opposing effects on the lower-affinity receptors that mediate inhibition of secretion. For reasons of simplicity, we will consider caerulein an agonist and CCK-JMV-180 an antagonist at those inhibitory receptors.

***In Vivo* Amylase Secretion.** The effects of caerulein and CCK-JMV-180 on *in vivo* secretion of amylase from rat pancreas are shown in Fig. 2. Caerulein was found to cause dose-dependent stimulation and, at supramaximally stimulating doses, inhibition of amylase secretion. Thus, caerulein at 0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$ caused a maximal rate of amylase secretion,

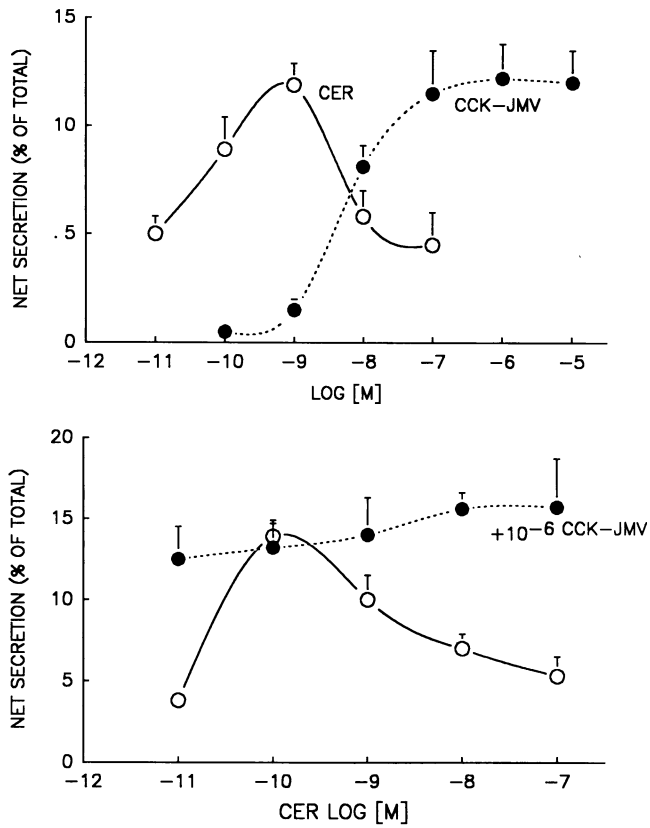

FIG. 1. Effect of caerulein and CCK-JMV-180 on amylase secretion. (*Upper*) Results obtained when rat pancreatic acini were incubated for 30 min in the presence of different concentrations of caerulein (CER) (○) or CCK-JMV-180 (●). (*Lower*) Results obtained when rat pancreatic acini were incubated in the presence of different concentrations of caerulein alone (○) or in combination with 1 μM CCK-JMV-180 (●). Results shown are mean values for 10–15 determinations in three separate experiments in which the replicate measurement varied by less than ±5%.

while caerulein at 5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$ inhibited amylase secretion. A maximal rate of amylase secretion was also observed when CCK-JMV-180 at 0.2 $mg \cdot kg^{-1} \cdot hr^{-1}$ was infused but, in this case, infusion of higher doses (5 $mg \cdot kg^{-1} \cdot hr^{-1}$) of CCK-JMV-180 did not inhibit secretion. The inhibition of secretion that followed infusion of a supramaximally stimulating dose of caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) could be prevented by the simultaneous infusion of a supramaximally stimulating dose of CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$). These *in vivo* observations are similar to those described above that were noted during *in vitro* secretion experiments.

Experimental Pancreatitis. Infusion of a supramaximally stimulating dose of caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) for 3.5 hr caused hyperamylasemia and pancreatic edema (Fig. 3) as well as acinar cell vacuolization and an inflammatory cell infiltrate in the pancreas (Table 1). Supramaximal stimulation with caerulein was also found to cause lysosomal enzyme redistribution and, as a result, a larger proportion of cathepsin B was isolated from the subcellular fraction enriched in zymogen granules (data not shown). These changes have been previously reported from this as well as other laboratories (1, 2, 13).

Based on the results of both *in vivo* and *in vitro* secretion studies (Figs. 1 and 2), we concluded that the CCK receptor affinity for CCK-JMV-180 was ≈1000-fold less than that for caerulein. To test the ability of CCK-JMV-180 to induce pancreatitis, rats were infused with that agent at a dose of 5 $mg \cdot kg^{-1} \cdot hr^{-1}$. No change in serum amylase activity or pancreatic water content was noted (Fig. 3). Similarly, no

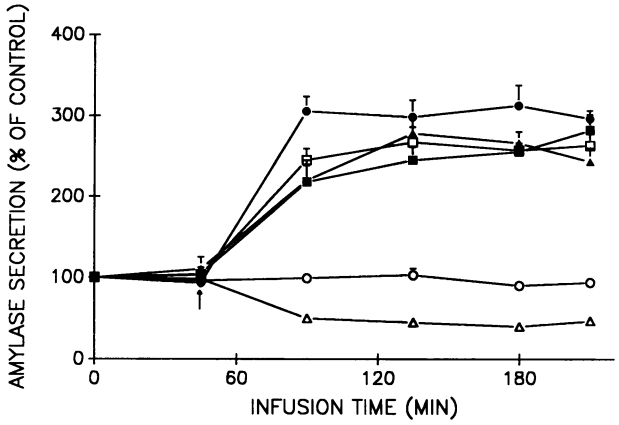

FIG. 2. Effect of caerulein and CCK-JMV-180 on *in vivo* amylase secretion. Rats were infused with heparinized saline alone (○) or saline containing caerulein (0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$; ●), caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$; △), CCK-JMV-180 (0.2 $mg \cdot kg^{-1} \cdot hr^{-1}$; ▲), CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$; □), or caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) plus CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$; ■). All animals were infused with saline alone for the first 30 min. Arrow indicates the start of infusion with various secretagogues. Amylase content in the pancreatic juice, collected every 30 min, was measured. Results are expressed as percent amylase activity present in the pancreatic juice at the beginning of infusion. Results shown are mean values of duplicate measurements obtained from at least three rats at each time point.

microscopic evidence of pancreatitis (i.e., vacuolization of acinar cells, inflammatory cell infiltrate) (Table 1) and no cathepsin B redistribution (data not shown) was noted after CCK-JMV-180 infusion. Infusion of CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$) with caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was found to prevent caerulein-induced hyperamylasemia and pancreatic edema (Fig. 3). as well as acinar cell vacuolization and inflammatory cell infiltration (Table 1). In addition, the subcellular redistribution of cathepsin B caused by caerulein infusion was prevented when CCK-JMV-180 was also given (data not shown). Infusion of a maximally stimulating dose of caerulein (0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) either alone or with somatostatin (4 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) did not cause changes typical of edematous pancreatitis (Table 1).

DISCUSSION

The recent development of CCK analogs such as CCK-JMV-180, which can discriminate between high- and low-affinity CCK receptors, has enabled studies that may identify the cell biology and pathological events mediated by each of these classes of receptor. As noted in this and other reports, CCK-JMV-180 acts as an agonist at high-affinity stimulatory receptors but antagonizes the inhibitory effect of caerulein at the lower-affinity class of receptor. The observation that CCK-JMV-180 cannot induce pancreatitis and that it prevents caerulein-induced pancreatitis leads to the unambiguous conclusion that caerulein induces pancreatitis by acting as an agonist at the lower-affinity CCK receptors. Those low-affinity receptors have been implicated in CCK-induced stimulation of 2-deoxyglucose uptake and inhibition of aminoisobutyric acid uptake by pancreatic acinar cells (7). Other as yet unidentified cellular events may also be regulated by those receptors.

Over the past decade, we have reported the results of a series of studies designed to characterize the early cell biological changes that occur during the evolution of caerulein-induced acute edematous pancreatitis and we compared those changes with the events that characterize the early stages of acute hemorrhagic pancreatic necrosis induced by feeding a choline-deficient ethionine-supplemented

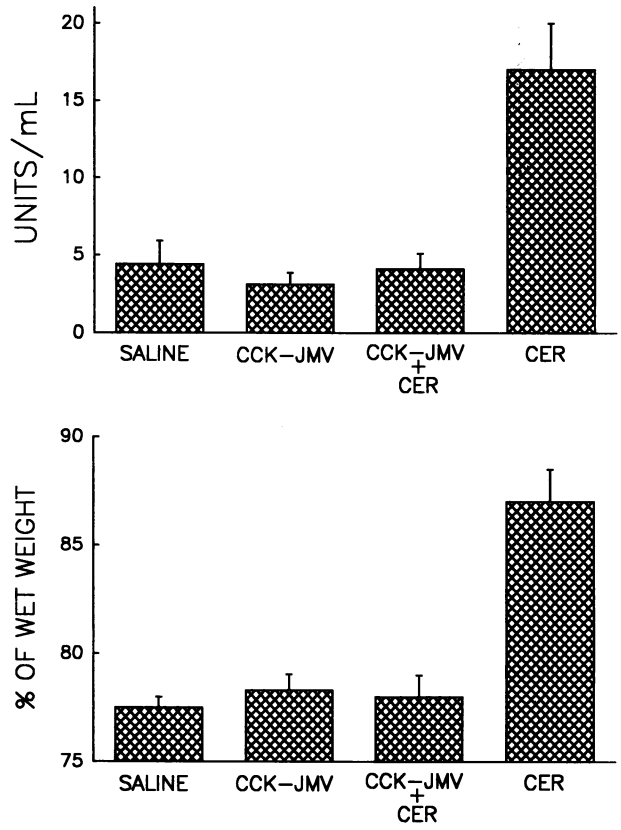

FIG. 3. Effect of CCK-JMV-180 on serum amylase level (*Upper*) and pancreatic water content (*Lower*). Rats were infused with heparinized saline alone (saline) or saline containing caerulein (CER; 5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$), CCK-JMV-180 (CCK-JMV; 5 $mg \cdot kg^{-1} \cdot hr^{-1}$), or a combination of caerulein and CCK-JMV-180 (CCK-JMV-CER) as described in the text. Serum amylase and pancreatic water content were independently measured on nine animals in each group and the results shown indicate the mean and SEM values for those measurements.

(CDE) diet to mice (1, 3, 13, 15–18). In both models of pancreatitis, the processes of amino acid uptake, protein and digestive enzyme synthesis, and intracellular transport of newly synthesized proteins to the zymogen granule compartment are not altered. Early in the development of both forms of experimental pancreatitis, however, digestive enzyme zymogens and lysosomal hydrolases become colocalized within large cytoplasmic vacuoles. This colocalization phenomenon, which is esterase dependent and which can be demonstrated by using morphological techniques of immu-

Table 1. Microscopic changes

Agent	*n*	Edema	Inflammatory cell infiltrate	Vacuolization
Saline	4	0	0	0
CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$)	4	0	0	0
Caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$)	8	4+	2+	4+
Caerulein (5 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) + CCK-JMV-180 (5 $mg \cdot kg^{-1} \cdot hr^{-1}$)	4	0	0	0
Caerulein (0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$)	4	0	0	0
Caerulein (0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) + somatostatin (4 $\mu g \cdot kg^{-1} \cdot hr^{-1}$)	4	0	0	0

Rats were infused with heparinized saline alone or containing various combinations of caerulein, CCK-JMV-180, and somatostatin for 3.5 hr as described in the text. Morphologic changes were graded on a 0–4+ scale by a "blinded" observer.

nolocalization, corresponds to the biochemically demonstrable redistribution of the lysosomal enzyme cathepsin B from the lysosomal-rich to the zymogen granule-rich subcellular fraction. In both models of pancreatitis, lysosomal enzyme redistribution is associated with increased lysosomal fragility. We have suggested (19) that the colocalization of digestive enzyme zymogens with lysosomal hydrolases may be of considerable pathophysiological importance because the lysosomal enzyme cathepsin B can activate trypsinogen (20), while trypsin can activate the remaining zymogens. Thus, colocalization of lysosomal enzymes with digestive zymogens could result in intra-acinar cell activation of digestive enzymes and the release of those activated enzymes within acinar cells.

An understanding of the mechanisms responsible for lysosomal and digestive enzyme colocalization during experimental pancreatitis may be of considerable value in the design of strategies to prevent or treat the clinical disease. In the CDE diet-induced model, a defect in stimulus–secretion coupling interferes with secretagogue-induced phospholipase C activation and 1,4,5-inositol trisphosphate generation (12). As a result, zymogen granules accumulate and fuse, by crinophagy, with lysosomes. In the caerulein-induced model of pancreatitis, colocalization appears to result from a combination of crinophagy and a defect in the normal sorting events by which digestive zymogens and lysosomal enzymes are segregated from each other as they pass through the Golgi apparatus. The observation, reported in this communication, that caerulein but not CCK-JMV-180 can induce edematous pancreatitis and lysosomal enzyme redistribution indicates that those phenomena result from low-affinity inhibitory receptor occupancy by an agonist but not by an antagonist. In other experiments, somatostatin (4 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) was infused with a maximally stimulating dose of caerulein (0.2 $\mu g \cdot kg^{-1} \cdot hr^{-1}$) to evaluate the possibility that the inhibition of caerulein-stimulated secretion by a ligand that does not interact with the low-affinity class of CCK receptors could also induce pancreatitis. In those studies, the simultaneous infusion of caerulein at a concentration that would have saturated the high-affinity stimulatory receptors combined with a dose of somatostatin sufficient to inhibit secretion did not cause hyperamylasemia, pancreatic edema, or acinar cell vacuolization (Table 1). These negative results suggest that caerulein-induced pancreatitis is dependent on the inhibition of secretion that results from low-affinity receptor occupancy and that other methods of inhibiting secretion may not be sufficient to induce pancreatitis.

In this communication, we have noted that CCK-JMV-180 can protect against caerulein-induced pancreatitis (Table 1, Fig. 3). In other studies, we have evaluated the possibility that CCK-JMV-180 might protect against caerulein-induced pancreatitis by mechanisms that do not involve antagonism of caerulein at low-affinity CCK receptors. In those studies (not shown), mice were fed a CDE diet and given subcutaneous injections of CCK-JMV-180 (5 mg per kg per 6 hr). No reduction in the mortality rate of CDE diet-induced pancreatitis was observed. The observation that CCK-JMV-180 fails to protect against pancreatitis induced by methods that do not involve CCK supports the conclusion that the protective effect of CCK-JMV-180 in caerulein-induced pancreatitis results from blockade of the low-affinity inhibitory receptors.

It has been generally assumed that clinical pancreatitis might be related to excessive pancreatic exocrine secretion. Support for this assumption has included the fact that the pancreas contains large quantities of potentially harmful digestive enzymes. In addition, the microscopic appearance of pancreatitis suggests that autodigestion of the gland has occurred. More convincing data implicating excessive secretion as a cause of pancreatitis, however, have not been reported. Attempts to treat clinical pancreatitis by reducing the stimulus for pancreatic secretion by gastric drainage (21) or administration of agents such as glucagon (22) or H_2 histamine receptor antagonists (23) have not been noted to favorably affect the course of the disease. Our observations using the caerulein and CDE diet-induced models of pancreatitis may redirect thinking in this area since they suggest that some forms of inhibition of secretion might contribute to the development of pancreatitis rather than to its treatment.

We would like to thank Ms. E. Glazer for her superb secretarial assistance. This research was supported by National Institutes of Health Grants AM31914 and AM31396. A.S. was supported by National Institutes of Health Grant DK-31092.

1. Saluja, A. K., Saito, I., Saluja, M., Houlihan, M. J. & Powers, R. E. (1985) *Am. J. Physiol.* **249,** G702–G710.
2. Lampel, M. & Kern, H. F. (1977) *Virchows Arch. A Pathol. Anat.* **37,** 97–117.
3. Watanabe, O., Baccino, F. M., Steer, M. L. & Meldolesi, J. (1984) *Am. J. Physiol.* **246,** G457–G467.
4. Ohshio, G., Saluja, A., Leli, U., Sengupta, A. & Steer, M. L. (1989) *Pancreas*, in press.
5. Silverman, M., Ilardi, C., Bank, S., Kranz, V. & Lendvai, S. (1989) *Gastroenterology* **96,** 186–192.
6. Niederau, C., Ferrell, L. D. & Grendell, J. H. (1985) *Gastroenterology* **88,** 1192–1204.
7. Sankaran, H., Goldfine, I. D., Bailey, A., Licko, V. & Williams, J. A. (1982) *Am. J. Physiol.* **242,** G250–G257.
8. Villaneuva, M. L., Martinez, J., Bodanszky, M., Collins, S. M., Jensen, R. T. & Gardner, J. D. (1982) *Am. J. Physiol.* **242,** G416–G422.
9. Galas, M.-C., Lignon, P. F., Rodriguez, M., Mendie, C., Fulcrand, P., Laue, J. & Martinez, J. (1988) *Am. J. Physiol.* **254,** G176–G182.
10. Stark, S. A., Sharp, C. M., Sutliff, V. E., Martinez, J., Jensen, R. T. & Gardner, J. D. (1989) *Biochem. Biophys. Acta* **1010,** 145–150.
11. Gaisano, H. Y., Klueppelberg, N. G., Pinon, D. I., Pfenning, M. A., Powers, S. P. & Miller, L. J. (1989) *J. Clin. Invest.* **831,** 321–325.
12. Powers, R. E., Saluja, A. K., Houlihan, M. J. & Steer, M. L. (1986) *J. Clin. Invest.* **77,** 1668–1674.
13. Saluja, A. K., Hashimoto, S., Saluja, M., Powers, R. E., Meldolesi, J. & Steer, M. L. (1987) *Am. J. Physiol.* **251,** G508–G516.
14. Bernfeld, P. (1955) *Methods Enzymol.* **1,** 149–150.
15. Gilliland, E. L. & Steer, M. L. (1980) *Am. J. Physiol.* **239,** G418–G426.
16. Koike, H., Steer, M. L. & Meldolesi, J. (1982) *Am. J. Physiol.* **242,** G297–G307.
17. Saito, I., Saluja, A., Steer, M. L. & Meldolesi, J. (1987) *Am. J. Physiol.* **251,** G517–G526.
18. Ohshio, G., Saluja, A., Leli, U., Sengupta, A. & Steer, M. L. (1989) *Gastroenterology* **96,** 853–859.
19. Steer, M. L., Meldolesi, J. & Figarella, C. (1984) *Dig. Dis. Sci.* **29,** 934–938.
20. Greenbaum, L. M. & Hirschkowitz, A. (1961) *Proc. Soc. Exp. Biol. Med.* **107,** 74–76.
21. Field, B. E., Hepner, G. W., Shabot, M. M., Schwartz, A. A., State, D., Worthen, N. & Wilson, R. (1979) *Dig. Dis. Sci.* **24,** 339–344.
22. Medical Research Council Multicentre Trial (1977) *Lancet* **ii,** 632–635.
23. Regan, P. T., Malagelada, J. R., Go, V. L. W., Wolf, A. M. & Di Magno, E. P. (1981) *Mayo Clin. Proc.* **56,** 499–503.