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PROTECTIVE EFFECT OF CALCITONIN GENE-RELATED PEPTIDE AGAINST CAERULEIN-INDUCED PANCREATITIS IN RATS

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The stimulation of sensory nerves by capsaicin exhibits the protective effect against caerulein-induced pancreatitis whereas deactivation of these nerves aggravates pancreatic damage evoked by overdose of caerulein. Calcitonin-gene related peptide (CGRP) has been identified as the prominent mediator of sensory nerves. The aim of the present study was to examine the influence of CGRP on the course of caerulein-induced pancreatitis (CIP). CIP led to a significant decrease in DNA synthesis and pancreatic blood flow (PBF) by 48% and 50% respectively, as well as a significant increase of pancreatic weight, plasma amylase concentration and development of the histological signs of pancreatic damage expressed as edema, leukocyte infiltration and vacuolization. Treatment with CGRP (2×10 μg/kg s.c.) attenuated the pancreatic tissue damage in caerulein-induced pancreatitis and completely reversed the deleterious effect of the ablation of sensory nerves on caerulein-induced pancreatitis. We conclude that CGRP exerts protective effect against caerulein-induced pancreatitis and is able to reverse the damage caused by deactivation of sensory nerves. Vasodilatation and preservation of pancreatic blood flow are involved in this effect.

Key words: *sensory nerves, capsaicin, pancreatic blood flow*

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a 37 amino acid molecule discovered primary in the thyroid as the product of an alternative processing of calcitonin gene (1). The gene has been shown to generate two different messenger RNAs, encoding either calcitonin or CGRP (2). CGRP is widely distributed throughout the central, peripheral and enteric nervous systems (3, 4). Within the enteric nervous system, CGRP-containing nerves have been found in large numbers among others in the stomach (5, 6), the intestine (4, 6)

and the pancreas (5—7). CGRP has numerous effects on gastrointestinal tissues including potent vasodilatation (8) and inhibition of gastric acid (5, 9) and pancreatic secretion (9—11). Intravenous administration of CGRP exerts protective effects in different experimental models of gastric lesions (12).

Immunocytochemical studies indicate that CGRP immunoreactivity is localized in the nerve fibres innervating the gastrointestinal tract and the pancreas, in the enteric ganglion cells of the intestine and in a subpopulation of cells of the islets of Langerhans (13, 14).

Capsaicin-sensitive afferent fibres in various pathophysiological aspects are implicated in the stomach and the pancreas, and CGRP is identified as a prominent mediator of these fibers. Treatment with capsaicin in high, systemic dose causes degeneration of most of the small diameter sensory neurons and leads to a significant depletion of CGRP content in the gastrointestinal tract and unmyelinated sensory fibres. It also decreases plasma CGRP content (15, 16), whereas, low dose of capsaicin stimulates the release of CGRP (17).

Stimulation of afferent neurons by intragastric administration of capsaicin induces a gastroprotection against damage caused by a variety of ulcerogenes (18, 19), while the capsaicin ablation of sensory neurons leads to an aggravation of gastric mucosal lesions (20, 21) and prolongs the ulcer healing (22). Similar effect of capsaicin on tissue integrity was observed in the pancreas (23). Activation of sensory fibres by capsaicin, attenuated the pancreatic damage in caerulein induced pancreatitis, whereas deactivation of afferent neurons by pretreatment with high doses of capsaicin contributed to the enhanced severity of pancreatitis.

The purpose of the present study was to determine the influence of CGRP on the maintenance of pancreatic integrity under normal conditions and in caerulein-induced pancreatitis. In addition, we tested whether exogenous CGRP would reverse deleterious effects of sensory denervation on the pancreas.

MATERIALS AND METHODS

Animals and treatment

Studies were performed on male Wistar rats weighing 160—190 g. Animals were housed in cages with wire mesh bottoms at room temperature with a 12 hour light, dark cycle. Water and food were available *ad libitum.*

Several series of experiments were carried out including: [1] control (0.9% NaCl s.c.); [2] caerulein induced pancreatitis; [3] capsaicin 100 mg/kg s.c. (ablatory dose of capsaicin); [4] CGRP 2×10 μg/kg s.c. (first injection 30 min before the start of the experiment; second 3 h later); [5] capsaicin-induced sensory nerve denervation + CGRP 2×10 μg/kg; [6] sensory fibres

denervation caused by capsaicin (100 mg/kg) + caerulein-induced pancreatitis; [7] caerulein-induced pancreatitis + CGRP (20 μg /kg of CGRP given s.c. in two doses: 10 μg/kg 30 min prior to caerulein infusion and 10 μg /kg 3 h later); [8] sensory fibres denervation caused by capsaicin + caerulein-induced pancreatitis + CGRP (given as in seventh group).

Sensory fibres denervation was induced by capsaicin in a total dose of 100 mg/kg, which was given in six injections (2.5+10+12.5+25+25+25 mg/kg s.c.) over 3 consecutive days. Two injections per day were performed in rats under ether anesthesia and a recovery period of 10 days was allowed before further experiments. To assess the effectiveness of sensory denervation, the day before the induction of pancreatitis, a drop of capsaicin (0.33 mM) was instilled into rat eye, and animals showing any wiping movements were excluded from the study.

Pancreatitis was induced by caerulein that was diluted in saline and infused s.c. for 5 h in conscious animals at a dose 10 μg/kg/h and at a rate of 1 ml/h.

Determination of pancreatic blood flow

After infusion of caerulein for 5 h, the animals were anesthetized with ether, weighed and the abdominal cavity was opened. The pancreas was exposed for the measurement of the blood flow in the pancreatic tissue by laser Doppler flowmeter using PeriFlux 4001 Master monitor (Perimed AB, Järfälla, Sweden). Blood flow was measured in five different portions of the pancreas. The pancreatic blood flow was presented as percent change from control value obtained in rats infused with saline.

Determination of plasma amylase concentration

Immediately after measurement of pancreatic blood flow the abdominal aorta was exposed and blood was taken for plasma amylase determination. Plasma amylase was determined by an enzymatic method (Amylase reagent, Dialab Diagnostic Ges. MBH, Wien, Austria). The values were expressed as units/liter.

Determination of DNA synthesis and RNA, DNA, protein content

After blood with drawal the pancreas was carefully dissected from its attachment to the stomach, the duodenum and the spleen. Fat and excess tissue were trimmed away. The pancreas was rinsed with saline, blotted on paper and weighed. The rate of DNA synthesis in the portion of minced pancreatic tissue was determined by incubating the tissue at 37°C for 45 min in 2 ml of medium containing 8 μCi/ml of [^{3}H]thymidine [6-^{3}H]-thymidine, 20—30 Ci/mmol, Institute for Research, Production and Application of Radioisotopes, Prague, Bohemia). The reaction was stopped with 0.4 N perchloric acid containing carrier thymidine (5 mM). Tissue samples were centrifuged and the precipitate washed twice in cold 0.2 N perchloric acid and recentrifuged. RNA was hydrolyzed in 0.3 M KOH incubated for 90 min at 37°C. DNA and protein were reprecipitated with 10% perchloric acid. After standing for 10 min on ice, the tubes were centrifuged and RNA content of the supernatant was measured using orcinol reaction (24). DNA in the residual pellets was solubilized in 10 % perchloric acid by heating at 70°C for 20 min. Denaturated protein was removed by centrifugation for 20 min. Using calf thymus as a standard, the DNA content of the samples was determined by Giles and Myers procedure (25). The final pellet was solubilized in 1 M NaOH and its protein content was determined by the Lowry method (26). The incorporation of [^{3}H]thymidine into DNA was determined by counting 0.5 ml DNA-containing supernatant in

a liquid scintillation system. RNA, DNA, protein contents were expressed as milligrams per pancreas. DNA synthesis was expressed as disintegrations per minute [^{3}H]thymidine per microgram DNA (dpm/μg DNA).

Histological examination

Samples of pancreatic tissue were excised, fixed in 10% formalin, embedded in paraffin and sections were stained with hematoxilin and eosin. The slides were examined histologically by two experienced pathologists without the knowledge of the treatment given. The histological grading of edema was made using a scale raging from 0 to 3; 0 = no edema, 1 = interlobular edema, 2 = interlobular and moderate intralobular edema, and 3 = interlobular edema and severe intralobular edema. Leukocytic infiltration was also graded from 0 (absent) to 3 for maximal alterations (diffuse infiltration in the entire pancreatic gland) Grading of vacuolization was based on the appropriate percentage of cells involved: 0 = absent, 1 = less than 25%, 2 = 25—50% and 3 = more than 50%.

Statistical analysis

Results are expressed as means ± S.E.M. and were analyzed by analysis of variance and Student's t test for unpaired values, with $p > 0.05$ considered significant.

RESULTS

Subcutaneous infusion of caerulein at a dose 10 μg/kg/h for 5 hours resulted in the formation of acute pancreatitis in all tested rats. The pancreas was swollen and enlarged with visible collection of edematous fluid. The weight of the pancreas was increased by 56% (*Table 1*) but RNA and DNA contents were not changed. DNA synthesis was decreased by 48% (*Fig. 1*) and the pancreatic protein content increased by 39% (*Fig. 2*). The plasma amylase concentration was ten fold increased above the value observed in control animals (*Fig. 3*). Pancreatic blood flow was reduced by 50% (*Fig. 4*). Histologically, infusion of caerulein always caused the interlobular, moderate

Table 1. Eeffect of saline (control, caerulein, capsaicin and CGRP given alone or in combination on pancreatic weight, RNA and DNA content.

	Pancreatic weight (mg)	RNA content (mg/pancreas)	DNA content (mg/pancreas)
Control	847.8 ± 32.7	7.62 ± 0.30	4.28 ± 0.12
Caerulein	1321.1 ± 70.2 [a]	7.12 ± 0.25	4.20 ± 0.13
Capsaicin	800.0 ± 42.0	6.24 ± 0.26 [a]	3.76 ± 0.17
CGRP	912.2 ± 52.7	8.50 ± 0.23	4.56 ± 0.20
Capsaicin + CGRP	810.0 ± 56.0	6.44 ± 0.21 [a]	3.79 ± 0.14
Capsaicin + Caerulein	1489.5 ± 80.1 [a]	6.17 ± 0.23 [a]	3.78 ± 0.16
CGRP + Caerulein	1069.3 ± 51.0 [a,b]	8.43 ± 0.28 [b]	4.57 ± 0.13
Capsaicin + CGRP + Caerulein	1261.0 ± 82.5 [a]	8.13 ± 0.32 [c]	4.33 ± 0.15

Mean ± S.E.M. of 8—10 rats. [a] $P < 0.05$ compared with control. [b] $P < 0.05$ compared with caerulein alone. [c] $P < 0.05$ compared with capsaicin given in combination with caerulein.

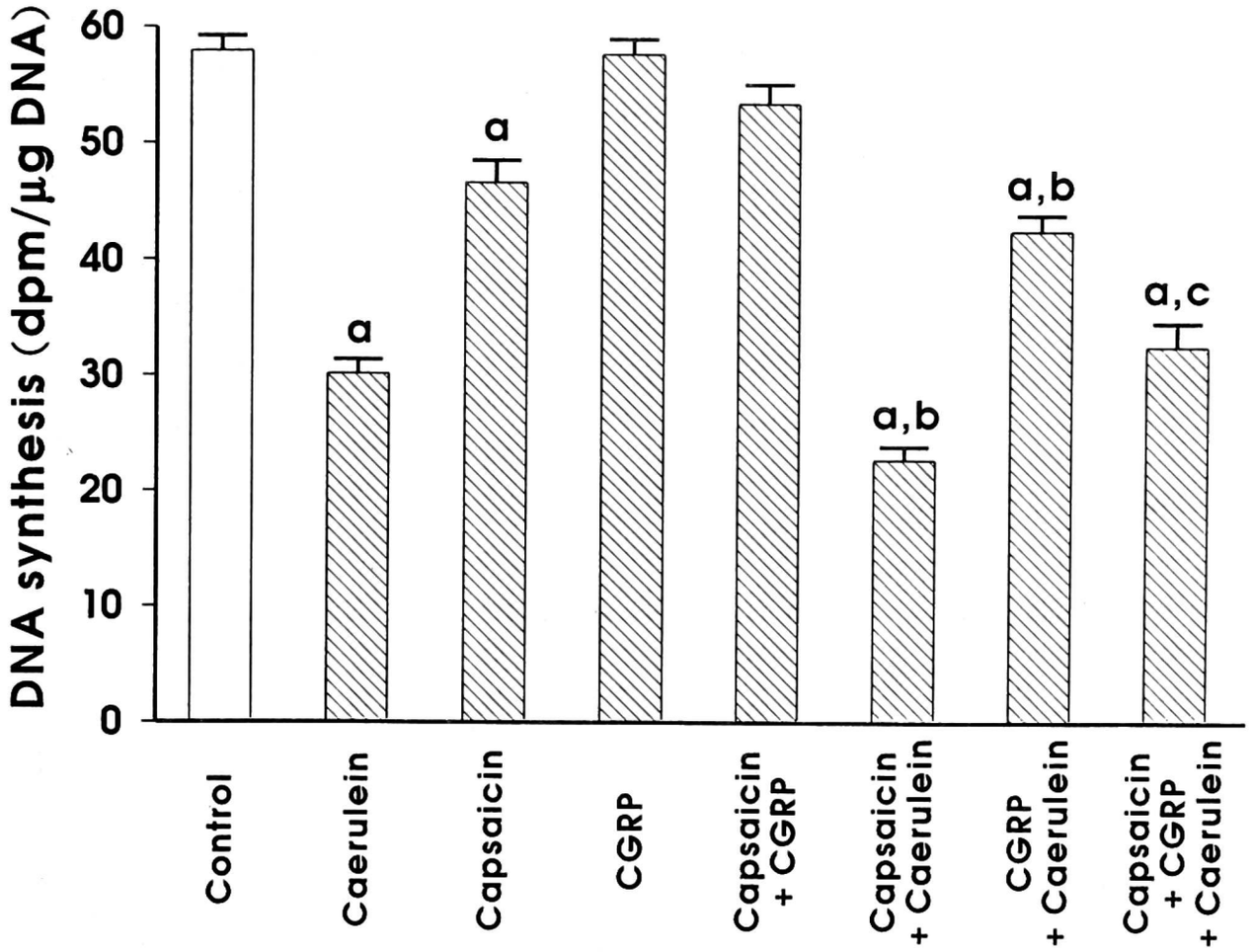

Fig. 1. Effect of saline (control), caerulein, neurotoxic dose of capsaicin and CGRP given alone or in combination on DNA synthesis in the pancreas. Mean ± S.E.M. of 8—10 observations. $^{a}P > 0.05$ compared with control, $^{b}P > 0.05$ compared with caerulein given alone, $^{c}P > 0.05$ compared with capsaicin given in combination with caerulein.

intralobular and in one third cases severe intralobular edema. The edema was accompanied by perivascular infiltration by leukocytes and the presence of vacuolization in about half of acinar cells (*Table 2*).

Table 2. Histological examination of pancreatic tissue after administration of saline (control), caerulein, capsaicin and CGRP alone or in combination.

HISTOLOGY			
	Edema (0—3)	Infiltration (0—3)	Vacuolization (0—3)
Control	0.60 ± 0.16	0.10 ± 0.10	0.00 ± 0.00
Caerulein	2.33 ± 0.23 [a]	1.77 ± 0.15 [a]	2.00 ± 0.24 [a]
Capsaicin	0.66 ± 0.21	1.00 ± 0.00	0.00 ± 0.00
CGRP	0.16 ± 0.16	0.00 ± 0.00	0.00 ± 0.00
Capsaicin + CGRP	0.66 ± 0.21	0.00 ± 0.00 [b]	0.00 ± 0.00
Capsaicin + Caerulein	2.71 ± 0.18 [a]	2.00 ± 0.00 [a]	2.71 ± 0.18 [a]
CGRP + Caerulein	1.43 ± 0.20 [c]	0.57 ± 0.20 [c]	1.85 ± 0.34 [a]
Capsaicin + CGRP + Caerulein	1.83 ± 0.30 [a]	1.16 ± 0.16 [a,b]	1.66 ± 0.21 [a,d]

Mean ± S.E.M. of 8—10 rats. $^{a}P < 0.05$ compared with control. $^{b}P < 0.05$ compared with capsaicin alone. $^{c}P < 0.05$ compared with caerulein alone. $^{d}P < 0.05$ compared with capsaicin given in combination with caerulein.

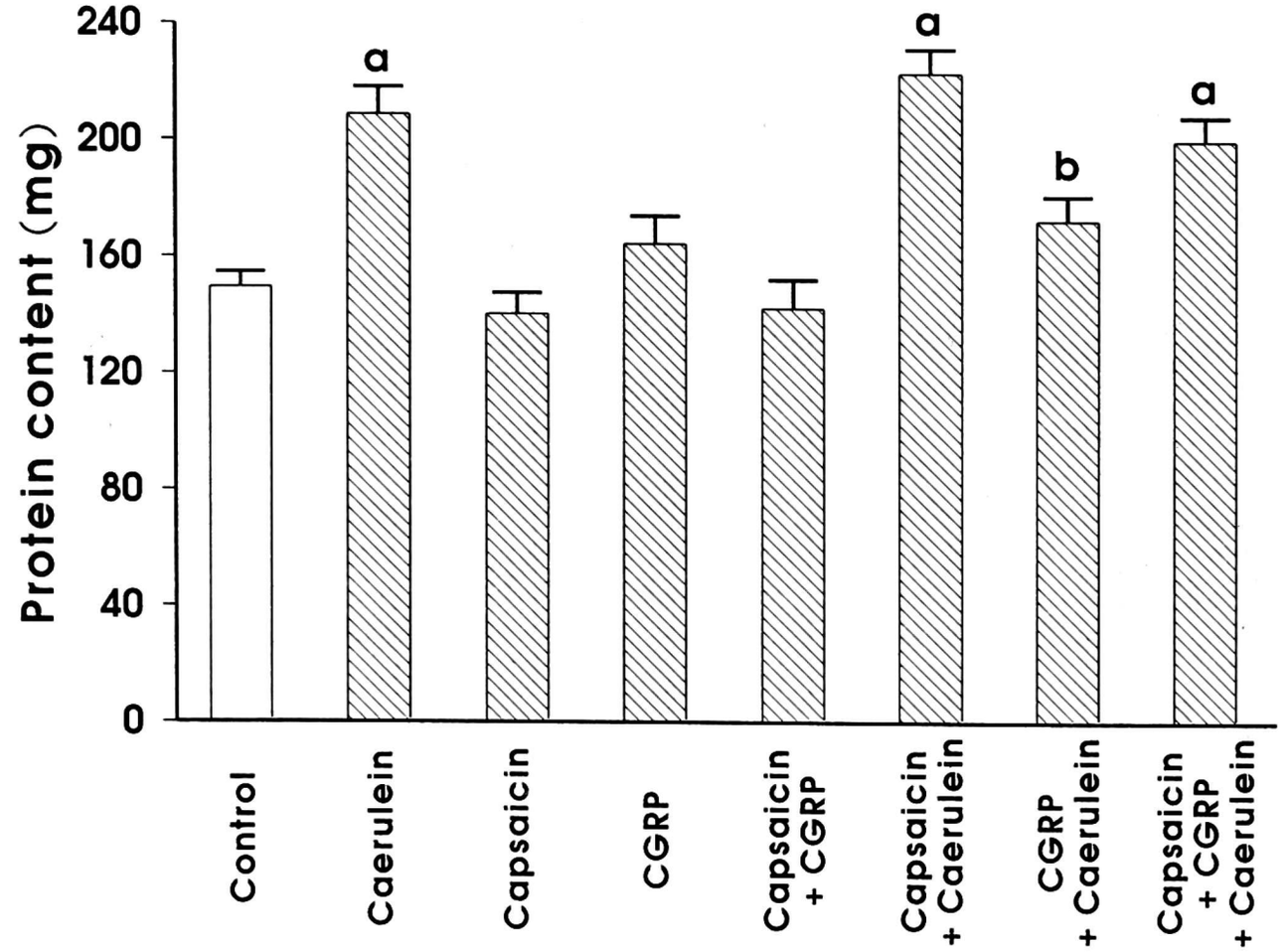

Fig. 2. Effect of saline (control), caerulein, neurotoxic dose of capsaicin and CGRP given alone or in combination on pancreatic protein content. Mean ± S.E.M. of 8—10 observations. [a]P > 0.05 compared with control, [b]P > 0.05 compared with caerulein given alone.

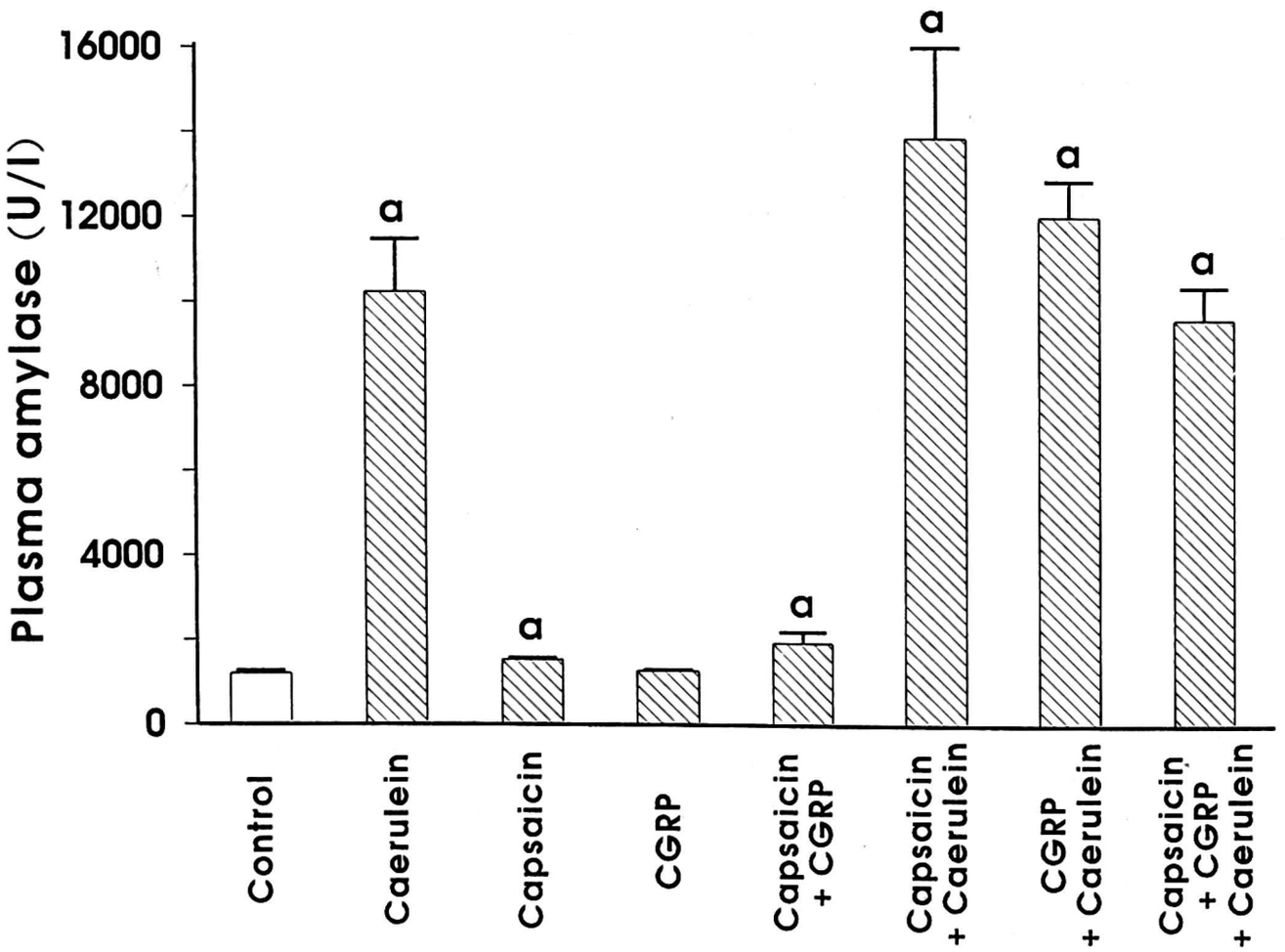

Fig. 3. Effect of saline (control), caerulein, neurotoxic dose of capsaicin and CGRP given alone or in combination on plasma amylase concentration. Mean ± S.E.M. of 8—10 observations. [a]P > 0.05 compared with control.

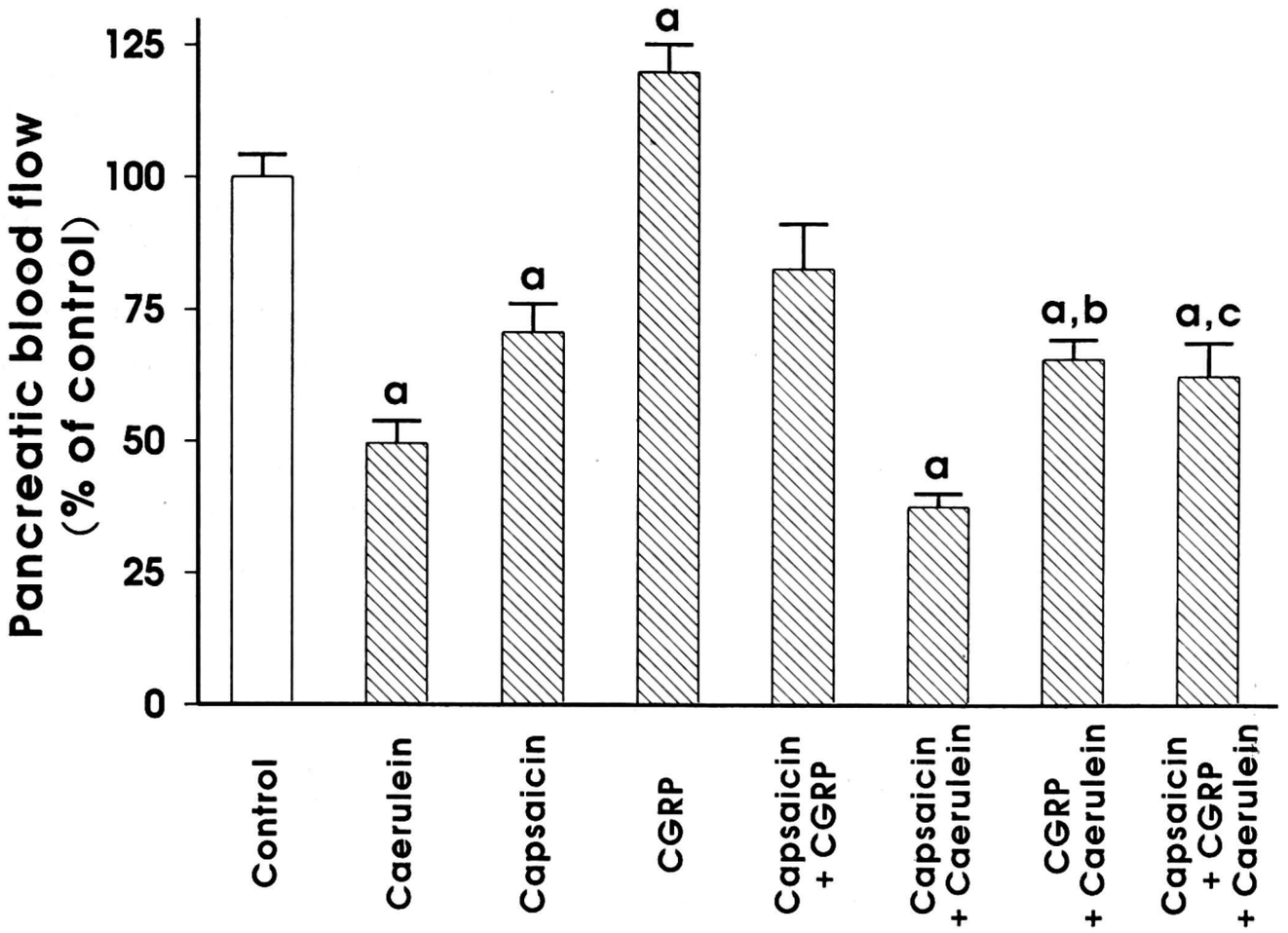

Fig. 4. Effect of saline (control), caerulein, neurotoxic dose of capsaicin and CGRP given alone or in combination on pancreatic blood flow. Mean ± S.E.M. of 8—10 observations. [a]$P > 0.05$ compared with control, [b]$P > 0.05$ compared with caerulein given alone, [c]$P > 0.05$ compared with capsaicin given in combination with caerulein.

Ablation of sensory nerves by capsaicin caused a significant decrease in RNA content, DNA synthesis and pancreatic blood flow. Plasma amylase concentration showed a small but significant increase (*Fig. 3*). No changes were observed in another biochemical parameters. Histological examination has shown that ablation of sensory nerves produced slight leukocyte infiltration without edema or vacuolization (*Table 2*). CGRP given alone caused a significant increase of pancreatic blood flow by 20% over the pretreatment value (*Fig. 4*), whereas other parameters were not significantly affected. CGRP in combination with ablation of sensory nerves was without effect on pancreatic weight, DNA content and also did not significantly affect a decrease in RNA content evoked by capsaicin (*Table 1*). The capsaicin-induced reduction of pancreatic DNA synthesis (*Fig. 1*) and pancreatic blood (*Fig. 4*) flow were almost completely reversed by the administration of CGRP. Pancreatic protein content was not affected by the combination of CGRP with capsaicin, whereas plasma amylase was above the control value or value observed after CGRP or capsaicin given separately. Morphological features (*Table 2*) have shown that CGRP prevented perivascular leukocyte infiltration induced by capsaicin. Capsaicin deactivation of sensory nerves prior to

caerulein infusion aggravated pancreatic damage created by caerulein, what was manifested by an additional significant decrease of RNA content, DNA synthesis and pancreatic blood flow. Histological examination revealed interlobular edema in all cases and the severe intralobular oedema was observed almost in all animals. Also leukocytic infiltration and vacuolization were more pronounced than after caerulein alone, but these changes as well as an increase of pancreatic weight, pancreatic protein content and plasma amylase concentration were not significantly different when compared with caerulein infusion.

Treatment with CGRP during caerulein infusion attenuated the severity of pancreatitis. The increase of pancreatic weight and pancreatic protein content was reduced when compared with caerulein alone but these parameters were still higher than in control group (*Table 1*). RNA content was increased and caerulein-induced a drop of DNA synthesis (*Fig. 1*) and pancreatic blood flow (*Fig. 4*) was partly, but significantly reversed. Also morphological features showed improvement of pancreatic histology, edema was limited to interlobular space in most cases and leukocytic infiltration was strongly reduced. Unexpectedly, plasma amylase concentration was even insignificantly higher than after caerulein alone (*Fig. 3*).

Deleterious effect of the ablation of sensory nerves on caerulein induced pancreatitis was completely reversed by CGRP administration. RNA content, DNA synthesis and pancreatic blood flow significantly increased and tended to reach higher values than after caerulein given alone. Histologically, the pancreatic condition was better than after caerulein given alone and leukocytic infiltration and vacuolization were significantly lower when compared with combination of capsaicin plus caerulein.

DISCUSSION

Our study provided evidence that CGRP can reduce pancreatic damage caused by caerulein hyperstimulation of the pancreas. Infusion of supramaximal doses of caerulein into rats (27), mice (28) and healthy human subjects (29) are known to induce acute edematous pancreatitis. Administration of caerulein results in a marked reduction of normotypic discharge of zymogen granules at the luminal plasma membrane (30). An ectopic discharge of individual granules and vacuoles is observed in lateral plasma membrane (30). Using immunocystochemical techniques, the presence of both secretory enzymes and lysosomal hydrolases has been demonstrated in these vacuoles (31, 32). An enhanced lysosomal degradation of cellular organelles and the free proteolitic activity most likely represents the crucial factor for further destruction of acinar cells (29, 33). These changes result in an induction of acute

pancreatitis which was manifested in our study as an inter- and intralobular edema, vacuolization of acinar cells and leukocytic infiltration. The pancreatic weight, pancreatic protein content and plasma amylase concentration were increased. The increase of pancreatic weight and protein content after induction of pancreatitis is probably due to the edema of pancreatic tissue and the leak of fluids and plasma proteins from blood vessels to interstitial pancreatic tissue. Also, in the caerulein-induced pancreatitis the pancreatic blood flow was strongly depressed and this effect was combined with the drop of DNA synthesis. The fall in the pancreatic blood flow seems to play an important role in the induction of pancreatic damage. As was observed earlier, the reduction of pancreatic blood flow can be also responsible for creation of acute pancreatitis by itself (34). However, it is a question whether changes in pancreatic blood flow, in most of the cases, are the cause of pancreatitis or represent a secondary phenomenon occurring as a consequence of acinar cell damage, intracellular activation of digestive enzymes and activation of inflammatory mediators.

Administration of CGRP during infusion of caerulein has exhibited a protective effect against pancreatic damage. Morphological features, as well as, biochemical parameters have shown an improvement of pancreatic tissue condition. However, it must be pointed out that one of the most accepted markers of pancreatic tissue damage, a plasma amylase concentration, has not been decreased and even insignificantly elevated. This inconsistency between pancreatic condition and plasma amylase concentration can be explained by smaller drop of pancreatic blood flow when caerulein infusion was combined with CGRP administration. The improvement of pancreatic blood flow allows for the removal of active digestive enzymes from pancreatic tissue and protects the pancreas against the damage caused by these enzymes. For the same reason plasma amylase concentration remains increased. These data indicates that beneficial effect of CGRP in caerulein-induced pancreatitis is dependent on improvement of pancreatic blood flow. Another helpful mechanism of CGRP action can be dependent on the inhibition of exocrine pancreatic secretion (10). Debas *et al.* (35) have suggest that CGRP evoked inhibition of exocrine pancreatic secretion is indirect, neurally mediated and may be explained by the release of somatostatin. This conception can be supported by studies showing favorable effect of somatostatin on the course of acute pancreatitis in animals (36) and people (37).

Previously, we have observed that stimulation of sensory afferent nerves shows a protective effect against acute inflammation induced by caerulein and ameliorates the biochemical manifestation of pancreatic damage. This effect was connected with an increase of pancreatic blood flow (23).

In the present study, we have observed the decrease in DNA synthesis, RNA content and pancreatic blood flow in test with ablation of capsaicin

sensitive sensory nerves without induction of pancreatitis. The role of sensory nerves has been more pronounced in pathological condition during caerulein-induced pancreatitis. Ablation of sensory nerves by high dose of capsaicin potentates the inhibition of pancreatic blood flow caused by caerulein and increases the severity of pancreatitis.

Deleterious effect of the ablation of sensory nerves on the course of caerulein-induced pancreatitis has been completely reversed by exogenous CGRP and the pancreatic condition has been even better than after caerulein given separately. These effects of CGRP on maintenance of pancreatic integrity are similar to effects observed after stimulation of sensory nerves (23). This observation and the information that low doses of capsaicin stimulate the release of CGRP (17) demonstrate that protective effects of sensory nerve stimulation in pancreas is to a high degree dependent on CGRP release. Additional support for this hypothesis is the finding that administration of a neurotoxic dose of capsaicin causes the persistent decrease in tissue CGRP-like immunoreactivity (38).

The protective effect of CGRP against caerulein-induced pancreatitis can be also dependent on the release of nitric oxide (NO). Interaction between release and action of CGRP and NO is unclear. Some previous reports have suggested that release of CGRP is NO dependent (39, 40), others have suggested that CGRP acts by NO release (41, 42). In addition, it has been shown by Tan *et al.* (43) that CGRP increases the activity of NO synthase. Both CGRP and NO cause vasodilatation (44) and the stimulation of afferent sensory nerves results in the release of endogenous CGRP (17) and NO (45). Furthermore, a reduction of NO synthesis by an inhibition of NO synthase aggravates the damage of the pancreas created by caerulein (46) and the degree of injury is almost the same as after sensory nerve ablation in combination with caerulein. Addition of L-arginine, a substratum for NO synthase, reverses deleterious effect of NO synthase inhibition (46).

On the second hand, overdose of NO participates in oxidative injury and contributes to multiorgan oxidative stress in pancreatitis (47) and NO may also reduce the antioxidant capacity of injured organs by binding the SH group (48). Moreover, NO can induce pancreatitis by itself (49). These findings have shown that the level of NO should to be within an appropriate range. Either excess or lack of NO can exhibit deleterious effect on pancreatic tissue. It is possible, that the stimulation of NO release by CGRP allows maintenance of a physiological amount of NO.

Prostaglandins were shown to reduce pancreatic edema, leukocytic infiltration and cellular necrosis in caerulein-induced pancreatitis (50, 51). In addition, the study of Hingtgen and Vasko (52) has shown that prostacyclin causes an increase in the resting and evoked release of substance P and CGRP from rat sensory neurons. Low concentrations of prostacyclin sensitize sensory

neurons to other stimuli such as low doses of capsaicin, bradykinin or hyperkaliemia, whereas higher concentrations may evoke release of neuropeptides directly. In such a case another possible mechanism of CGRP action can be dependent on interaction between CGRP and prostaglandins.

In summary, our study demonstrates that CGRP exhibits a protective effect against caerulein induced pancreatitis. Its beneficial action can be dependent on multiple mechanisms, in which an essential role plays a preservation of pancreatic blood flow.

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