REGULATION OF DUODENAL MUCOSAL BICARBONATE SECRETION

by

H. S. ODES, M.B.,Ch.B. (U.C.T.), F.C.P. (S.A.)

University of Cape Town

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DEDICATION

This work is dedicated to my wife,

Dr. Sandra Odes, M.B.,Ch.B. (U.C.T.),

without whose help, devotion, support and encouragement, the undertaking and completion of this project would not have been possible.

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PUBLISHED PAPERS ARISING FROM THIS RESEARCH
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Reimer R, Heim H-K, Muallem R, Odes HS, Sewing K-F.
Effects of EP-Receptor Subtype Specific Agonists and other Prostanoids on Adenylate Cyclase Activity of Duodenal Epithelial Cells.
Prostaglandins 44: 485-493, 1992.

Odes HS, Muallem R, Reimer R, Beil W, Schwenk M, Sewing K-Fr.
Cholinergic Regulation of Guinea-Pig Duodenal Bicarbonate Secretion.
American Journal of Physiology 265 (Gastrointestinal and Liver Physiology 28): G270-G276, 1993.

Muallem R, Reimer R, Odes HS, Schwenk M, Beil W, Sewing K-F.
Role of Carbonic Anhydrase in Basal and Stimulated Bicarbonate Secretion by the Guinea Pig Duodenum.
Digestive Diseases and Sciences - in press

Reimer R, Odes HS, Muallem R, Schwenk M, Beil W, Sewing K-F.
Cyclic AMP is the second messenger of PGE2- and VIP-stimulated active bicarbonate secretion by guinea-pig duodenum.
Scandinavian Journal of Gastroenterology - in press

ABBREVIATIONS

A23187, Ca^{2+} ionophore

cAMP, 5'-cyclic adenosine monophosphate

cGMP, 5'-cyclic guanosine monophosphate

dBcAMP, dibutyryl 5'-cyclic adenosine monophosphate

dBcGMP, dibutyryl 5'-cyclic guanosine monophosphate

DMSO, dimethyl sulfoxide

DNSA, dimethylamino-naphthaline-5-sulfonamide

i.v., intravenous

m_1 and m_2-cholinoceptor, m_1 and m_2 muscarinic receptors

n-cholinoceptors, nicotinic receptors

NS, not significant

PGE_2, prostaglandin E_2

$PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

PKA, protein kinase A

PKC, protein kinase C

TPA, tetradecanoyl-phorbol-acetate

VIP, vasoactive intestinal polypeptide.

[D-p-Cl-Phe^6,Leu^{17}]-VIP, competitive antagonist at VIP receptor

NOTE ON TERMINOLOGY: The word "agonist" is used for simplicity for all stimulants of bicarbonate secretion, although it is realized that the longer term "secretory stimulant" is more accurate, since some "agonists," like the cyclic nucleotides, do not have cell membrane receptors.

ABSTRACT

The present research studied the regulation of duodenal bicarbonate secretion in the anaesthetized guinea-pig, using a model that permitted the study of active transport of bicarbonate.

It was determined that dibutyryl 3',5'-cyclic adenosine monophosphate, vasoactive intestinal polypeptide, prostaglandin E_2, carbachol and theophylline are the chief agonists of duodenal bicarbonate secretion. Vasoactive intestinal polypeptide and prostaglandin E_2 act directly via distinct receptors on the duodenal enterocytes, activating adenylate cyclase and protein kinase A in sequence to initiate bicarbonate secretion.

In addition, there is good evidence that the inositol phospholipid and protein kinase C cascade is also involved, possibly to a lesser extent, since tetradecanoyl-phorbol-acetate and prostaglandin F_{2a} were agonists of bicarbonate secretion.

Carbachol, using a m-cholinoceptor pathway, stimulates duodenal bicarbonate secretion by releasing vasoactive intestinal polypeptide. Consistent with this finding is the observation that carbachol has no receptors on duodenal enterocytes. The role of the nicotinic pathway in bicarbonate secretion, however, remains uncertain.

Duodenal bicarbonate secretion can be inhibited by somatostatin and acetazolamide. Somatostatin selectively

suppresses carbachol-stimulated and VIP-stimulated duodenal bicarbonate secretion, but not PGE_2-stimulated bicarbonate secretion. Receptors for somatostatin coupled to adenylate cyclase could not be detected on isolated duodenal enterocytes, which strengthens the hypothesis that carbachol does not act directly on these epithelial cells, but via a second transmitter, vasoactive intestinal polypeptide.

Carbonic anhydrase activity is necessary for secretion of bicarbonate, since acetazolamide-inhibition of this enzyme decreased bicarbonate secretion, both basal and stimulated by many different agonists. Carbonic anhydrase serves as a common final step in the generation of bicarbonate in duodenal enterocytes. This enzyme was located in the cytoplasm of cells in the villus as well as the crypt cells, implying that bicarbonate secretion occurs along the length of the villus and crypt.

In summary, the present research has shown direct stimulation of duodenal bicarbonate secretion by vasoactive intestinal polypeptide, which participates also in the m-cholinergic pathway, and by prostaglandin E_2. Adenylate cyclase and protein kinase A appear to be the intracellular messengers with the primary function of initiating duodenal bicarbonate secretion. However, there is convincing evidence that the inositol phospholipid and protein kinase C cascade also activates this secretion. Somatostatin selectively stops duodenal bicarbonate secretion. Carbonic anhydrase

activity in the crypt and villus is required as the final common step in bicarbonate production.

OBJECTIVES OF THE RESEARCH

1. to set up and validate the methodology required for the study of active bicarbonate secretion in the guinea-pig duodenum in vivo
2. to determine and compare the dose-response relationships of the chief agonists of duodenal bicarbonate secretion
3. to define in detail the nature of the PGE_2, VIP and cholinergic pathways that activate duodenal bicarbonate secretion
4. to correlate the information obtained about extracellular factors regulating duodenal bicarbonate secretion with their corresponding intracellular messenger systems
5. to examine the inhibitory effects of somatostatin-14 peptide in relation to the PGE_2, VIP and cholinergic pathways that activate duodenal bicarbonate secretion
6. to examine the effect of inhibition (by acetazolamide) of carbonic anhydrase on the PGE_2, VIP, cholinergic and other pathways that stimulate duodenal bicarbonate secretion

INTRODUCTION

1. MUCOSAL BICARBONATE SECRETION AS A PHYSIOLOGICAL DEFENCE MECHANISM AGAINST PEPTIC ULCERATION IN THE PROXIMAL DUODENUM (THE DUODENAL BULB)

When the stomach empties after a meal, gastric acid enters the proximal duodenum and reduces the intraluminal pH rapidly to < 3. It is necessary that this acid load be neutralized in order that digestion will proceed normally. This function is served by bicarbonate secreted from the pancreas and the liver (into pancreatic juice and bile respectively).

There is however another function of bicarbonate in the duodenum, which is, to prevent gastric acid and pepsin from causing peptic ulceration of the duodenal mucosa. The duodenal mucosa is now known to secrete an alkaline fluid containing $NaHCO_3$; there is no hard evidence that Brunner's glands produce bicarbonate (Flemstrom 1987). Based on a body of experimental observations, it is hypothesized that the function of duodenal mucosal bicarbonate secretion is to alkalinize the unstirred mucous layer adherent to the mucosal epithelium and thus prevent penetration of acid. This idea was proposed by Kivilaakso & Flemstrom (1984). It follows then that marked deficiency of bicarbonate production in the duodenal bulb mucosa would allow

hydrochloric acid and pepsin to reach and destroy the mucosal epithelial cells and penetrate deeply into the lamina propria and beyond, to cause true peptic ulceration (Isenberg et al. 1991).

Several important experimental studies in normal men and women, and in persons with active and healed duodenal ulcers, provide compelling evidence in support of the hypothesis outlined here. These are reviewed briefly. Patients with duodenal ulcers secrete less bicarbonate from the duodenal bulb than normal persons in the basal (fasting) state as well as during duodenal lumen acidification (Isenberg et al. 1987, Hogan & Isenberg 1991). This deficiency of bicarbonate output is noted, too, when the ulcer disease is in remission and the duodenal bulb mucosa appears to be completely normal at endoscopy and by histological examination (Basuk et al. 1989). This abnormality of bicarbonate secretion appears to be related to defective synthesis or release of mucosal PGE_2 in duodenal ulcer patients (Bukhave et al. 1990, Hogan et al. 1990). Hydrochloric acid stimulates duodenal bicarbonate secretion; this secretion is reduced to basal values by indomethacin, which diminishes endogenous production of prostaglandins by the duodenum (Isenberg et al. 1985). Most interestingly, cigarette smokers, who have a clearly higher rate of chronic, recurrent duodenal ulcer disease, are found to have diminished basal as well as hydrochloric acid-

stimulated duodenal bicarbonate secretion (Isenberg et al. 1987, Hogan & Isenberg 1991).

One animal study also provides corroborative evidence for the hypothesis given above. In a classical rat model, cysteamine induced duodenal ulceration, which has some resemblance to human duodenal ulcer disease, is associated with strikingly reduced duodenal bicarbonate secretion (Stiel et al. 1983). It is interesting that only man and the horse Equus caballus are afflicted by chronic duodenal ulcer disease (Isenberg & Hogan, 1990). Abnormalities of bicarbonate production have hitherto been documented only in human subjects with duodenal ulcers, and not in animals.

The duodenal bulb is that region of the duodenum which is exposed to the lowest intraluminal pH. Of note, it is this organ which is particularly vulnerable to peptic ulceration. It is therefore not surprising that the duodenal bulb secretes very much more bicarbonate than the distal duodenum and jejunum, as shown in the rat (Isenberg et al. 1983) and confirmed in man (Isenberg et al. 1986).

In this thesis, bicarbonate secretion, directed against acid and pepsin from the stomach, is examined in a specific animal model. Other mechanisms that cause ulcers (which include Helicobacter pylori, genetic abnormalities, hypergastrinaemia and nicotine), and additional factors that protect the duodenum (mucus secreted by duodenal mucosal

cells and Brunner's glands, antero- and retrograde duodenal motility, etc.) are not examined here.

2. EXTRACELLULAR CONTROL OF DUODENAL BICARBONATE SECRETION BY GASTROINTESTINAL PEPTIDE HORMONES AND PROSTAGLANDINS

Duodenal bicarbonate secretion was likely to be a receptor-mediated event involving intracellular second messenger cascades.

Studies in the bullfrog, rat, guinea-pig, rabbit, cat, dog and man, in vivo and in vitro, showed that duodenal bicarbonate secretion is stimulated by prostaglandins E and F, and the hormones VIP and glucagon, as well as several other compounds (Table 1).

In addition, there is also a strong and very important element of autonomic neural control of bicarbonate secretion, parasympathetic as well as sympathetic (Fandriks et al. 1991); cholinergic regulation is considered in detail below.

The most potent agonist of duodenal bicarbonate secretion in mammals, including man, is hydrochloric acid, as shown in a number of studies where HCl was instilled intraluminally in the duodenum at a concentration up to 150 mmol/l (Flemstrom et al. 1982a, Isenberg et al. 1985, Isenberg et al. 1985). It should be appreciated that intraluminal acid is the natural stimulus to duodenal

TABLE 1. Bicarbonate secretion by the duodenal surface epithelium (partial list). Some of the described effects are species-specific or are controversial.

Stimulants:

dBcAMP, arachidonic acid, beta-endorphin, carbachol, enkephalins, gastric inhibitory polypeptide, morphine, neurotensin, norepinephrine, pancreatic glucagon, prostaglandins: E_1, E_2, $F_{2\alpha}$, theophylline, vasoactive intestinal polypeptide.

Inhibitors:

acetazolamide, anoxia, clonidine, cyanide, ouabain.

No effect:

carbachol, dBcGMP, gastrin, insulin, secretin, serotonin, substance P, urogastrone.

(from references: Algazi et al. 1989, Ballesteros et al. 1991, Fandriks et al. 1986, Fandriks et al. 1991, Flemstrom & Turnberg 1984, Flemstrom et al. 1993, Garner et al. 1990, Granstam et al. 1987, Hogan et al. 1991, Hurst et al. 1982, Isenberg et al. 1985, Isenberg & Hogan 1990, Knutson et al. 1992, Konturek et al. 1987, Lenz et al. 1989, Odes et al. in press, Safsten & Flemstrom 1986, Simson et al. 1981a, Simson et al. 1981b, Smedfors & Johansson 1986, Takeuchi et al. 1990)

bicarbonate secretion; the pH threshold for duodenal bicarbonate secretion in humans was investigated and found to be 3.0 (Feitelberg et al. 1992).

Acid-stimulated duodenal bicarbonate secretion was shown to be associated with the release of PGE_2 into the duodenal tissue and duodenal lumen, and this bicarbonate secretion was markedly reduced by pre-treatment with indomethacin (Isenberg et al. 1986, Selling et al. 1987). HCl-stimulated duodenal bicarbonate secretion also involves the release of the hormone VIP (Smedfors et al. 1987). Stimulation by VIP mimics HCl-induced bicarbonate secretion (Wolosin et al. 1989). Therefore, from these studies it appeared that certain prostaglandins, notably PGE_2, and hormones, in particular the neuropeptide VIP, play a critical role in the regulation of duodenal bicarbonate secretion following stimulation by HCl. How PGE_2 and VIP actually work was not clear. How other agonists work was also uncertain. It was not clear that all reputed agonists were really that. Therefore, while the extracellular regulation of bicarbonate secretion by the duodenal mucosa was well described, it was nonetheless incompletely understood.

There were problems with many studies in the literature. Passive diffusion of bicarbonate from the blood to the duodenal lumen was not distinguished from active transport of this anion. The blood-to-lumen gradient for bicarbonate in most experiments, where the duodenal

perfusate contained 154 mmol/l NaCl, was estimated as being approximately 25 : 1 (Isenberg & Hogan 1990). This shows therefore that "active" secretory phenomena were not being measured specifically. This does not mean, of course, that active secretion did not occur; it simply implies that this question was not being addressed. Of note, there was a strong "contrary" view that most bicarbonate "secretion" was really diffusion (Vattay et al. 1988).

Also, there was little clarity about the relative potency of the identified agonists of bicarbonate secretion, since different species were used (and species-differences were observed), and experimental conditions varied considerably. The multitude of agonists somewhat clouded the picture of what the true physiological agonists were likely to be.

3. INTRACELLULAR SIGNALLING MECHANISMS OF GASTROINTESTINAL PEPTIDE HORMONES AND PROSTAGLANDINS

The intracellular signalling mechanisms related to PGE_2 and VIP, as well as other reputed agonists, were little studied. Little was known about whether the described actions of the agonists were receptor-related events.

In early studies by Simson et al. (1981b), cAMP was suggested to act as a second messenger in bicarbonate secretion by frog duodenum in the Ussing chamber, since this secretion could be elicited by receptor-independent

adenylate cyclase activators like forskolin, by cAMP analogues and by theophylline. This fitted in nicely with data about the mode of action of PGE_2 and certain peptide hormones, like VIP, in other parts of the gastrointestinal tract (Smith et al. 1990). A postulated role for cAMP as a second messenger was confirmed more recently by Dunk et al. (1989), Garner et al. (1990) and Yao et al. (1993).

Knowledge concerning calcium as an intracelllular mediator of bicarbonate secretion was more speculative, since cholinergic stimulation did not appear to activate bicarbonate secretion in most species tested (Flemstrom et al. 1985, Takeuchi et al. 1990; see section on cholinergic regulation below).

In interpreting the data from studies of intracellular messengers in the literature, therefore, there remained several unclear issues, which are addressed here in this thesis.

Approach to these issues:

A series of experiments was designed to address the controversies described in this section of the research.

Studies of bicarbonate secretion were performed in the isolated, perfused proximal duodenum of the guinea-pig in vivo.

To begin with, compounds which activate the adenylate cyclase/cAMP/PKA and the inositol phospholipid/PKC cascades were tested in vivo under conditions of active (uphill) bicarbonate secretion. This was needed to obtain dose-response data to show the relative potency of the agonists of bicarbonate secretion, and also indicated which intracellular messengers were likely to be operative in the process of bicarbonate secretion.

Based on this information, selected doses of these agonists were next administered in combination in order to obtain further information relating to the intracellular mediators of bicarbonate transport.

These studies in the guinea-pig in vivo were then complemented by certain experiments in isolated duodenal enterocytes in vitro where, for example, the response of adenylate cyclase and protein kinase A was measured in homogenates of duodenal enterocytes during exposure to the same agonists used in vivo. This enabled the in vivo data to be correlated with the results from the in vitro studies.

The in vitro studies were performed in a collaborative project (see Acknowledgements) and are important for understanding the meaning of certain of the in vivo results.

4. CHOLINERGIC REGULATION OF DUODENAL BICARBONATE SECRETION

As indicated above, neural regulation of bicarbonate secretion is very important. The cholinergic system is stimulatory and the sympathetics are inhibitory, in general (Flemstrom 1987, Fandriks et al. 1991). In this work, the cholinergic system alone is investigated.

There are several studies that indicate that vagal stimulation, whether electrical (Granstam et al. 1987, Nylander et al. 1987), drug-induced (Lenz et al. 1989, Safsten & Flemstrom 1986) or by sham feeding (Ballesteros et al. 1991, Konturek & Thor 1987), promptly induces duodenal bicarbonate secretion in animals and in man.

It was therefore expected that this action can be mimicked by m-cholinoceptor (muscarinic receptor) agonists, and that this would provide a sound laboratory tool for studies of this regulation. However, experimental cholinergic manipulation of bicarbonate secretion in different species has produced the most conflicting data.

Carbachol, which acts on m-cholinoceptors and n-cholinoceptors (nicotinic receptors), and bethanechol, which

acts mainly on m-cholinoceptors (Goyal 1989), did not consistently increase duodenal bicarbonate output in man or animals (Ballesteros et al. 1991, Flemstrom et al. 1985, Lenz et al. 1989). Atropine, a non-specific antagonist at m_1- and m_2-cholinoceptors, inhibited basal but not sham-feeding stimulated bicarbonate secretion (Ballesteros et al. 1991, Fandriks 1986, Jonson et al. 1986, Lenz et al. 1989, Smedfors et al. 1990). These disclosures insinuated that cholinergic stimulation of bicarbonate secretion did not utilize the m-cholinoceptor pathways.

As an unexpected result, Safsten & Flemstrom (1986) reported that pirenzepine, an m_1-cholinoceptor antagonist (Goyal 1989), actually stimulated duodenal bicarbonate secretion in the rat in vivo.

The n-cholinoceptor antagonist hexamethonium strongly curbed the effect of vagal stimulation of bicarbonate secretion, although this property was associated with systemic hypotension which itself depresses bicarbonate secretion (Fandriks 1986, Granstam et al. 1987, Nylander et al. 1987). The exact role of n-cholinoceptors, in any, was thus in doubt.

Of note, there was good evidence that acetylcholine acts in the intestine by releasing the neuropeptide VIP (Cassuto et al. 1983)

In summary, then, the extracellular cholinergic control of duodenal bicarbonate secretion was incompletely understood, and so required definitive characterization. As

a consequence, little was known about the intracellular second messenger, in particular Ca^{2+}, of this cholinergic mechanism.

Approach to these issues:

The following series of experiments were performed to address these issues pertaining to the cholinergic control of bicarbonate secretion.

Dose-response data were obtained with carbachol to see whether this agent could be used to mimic cholinergic stimulation of bicarbonate secretion.

The effects of atropine, pirenzepine and hexamethonium were studied on basal and carbachol-stimulated bicarbonate secretion to get further information on the nature of the cholinergic pathway of duodenal bicarbonate secretion.

[D-p-Cl-Phe^{6},Leu^{17}]-vasoactive intestinal peptide, an antagonist to VIP, was tested in carbachol-stimulated bicarbonate secretion.

Verapamil, nifedipine and the calcium ionophore A23187 were employed to examine the role of intracellular Ca^{2+} in cholinoceptor mediated stimulation of bicarbonate. Verapamil and nifedipine were tested for their effects on carbachol-stimulated bicarbonate secretion. A23187 was tested dose-responsively to determine whether it could be used as an agonist of bicarbonate secretion. Furthermore, the effect of

combining A23187 with carbachol or with the VIP-antagonist was assessed.

An attempt was made in vitro to detect cholinoceptors on isolated duodenal enterocytes.

5. REGULATORY ROLE OF SOMATOSTATIN IN DUODENAL BICARBONATE SECRETION

The gastrointestinal organs, particularly the stomach and duodenum, contain 70% of the body's somatostatin (Yamada & Chiba 1989, Lucey & Yamada 1989, Francis et al. 1990). Somatostatin exists as 14 and 28 amino acid peptides, derived by different processing of the common precursor prosomatostatin. The duodenum contains more somatostatin-14, located in the peptidergic neural system, than somatostatin-28, found in the mucosa (Yamada & Chiba 1989).

While somatostatin was shown to act in paracrine fashion to inhibit most gastrointestinal secretory processes (Yamada & Chiba 1989), its role in the regulation of duodenal bicarbonate secretion was not well understood.

Somatostatin-14 had no effect on bicarbonate secretion by frog duodenum in vitro (Flemstrom et al. 1982b), and had variable effects on bicarbonate secretion by the perfused rat duodenum (Kirkegaard et al. 1984, Lenz & Forquignon 1990). Somatostatin-14 decreased VIP-stimulated bicarbonate

secretion in the perfused rat duodenum (Kirkegaard et al. 1984). Intravenous somatostatin-28 did not affect rat duodenal bicarbonate secretion (Lenz & Forquignon 1990).

The role of somatostatin in duodenal bicarbonate secretion required further definition. It was decided to study in detail the 14 amino acid peptide form, which appears to act in the enteric neural system and which is likely to be that form which regulated bicarbonate secretion.

Approach to these issues:

Experiments was performed to gain more information on the regulation of bicarbonate secretion by somatostatin-14.

The effects of somatostatin-14, carbachol, VIP and PGE_2 on bicarbonate secretion were tested in the perfused proximal duodenum of the guinea pig.

Then, the effects of these agents on adenylate cyclase activity in homogenates of duodenal enterocytes were measured to determine the mechanism of action of somatostatin in the duodenum.

6. FACILITATORY ROLE OF CARBONIC ANHYDRASE IN DUODENAL BICARBONATE SECRETION

The intracellular transport mechanisms of duodenal bicarbonate secretion are incompletely characterized. The

role of carbonic anhydrase, however, is germane in this context, since this enzyme is postulated by most workers to constitute the final common step in the production of bicarbonate by the duodenal enterocytes (other sources of bicarbonate are also possible, such as diffused bicarbonate in the frog).

This enzyme is present in most tissues and hydrates CO_2 to produce HCO_3^- and H^+ ions, a critical metabolic process in gastrointestinal acid/base transport (Gleeson 1992). Carbonic anhydrase is linked to ion exporters and exchangers at both the apical and basolateral cell membrane.

Acetazolamide is a convenient metabolic tool which reversibly inhibits carbonic anhydrase activity by a process of N-sulfamyl substitution. Results of studies using this drug to inhibit bicarbonate secretion were however not consistent.

In small mammals in vivo, the effect of acetazolamide was variable, and bicarbonate secretion was inhibited only when the basal rate of bicarbonate secretion was high, or when PGE_2 was used as an agonist (Flemstrom & Kivilaakso 1983, Holm et al. 1990, Isenberg et al. 1983, Takeuchi et al. 1986). In the bullfrog duodenum in vitro, inhibition of carbonic anhydrase by acetazolamide (5×10^{-4} mol/L) did not affect basal bicarbonate secretion, whereas a quite large dose of acetazolamide (10^{-2} mol/L) produced 30% inhibition of basal bicarbonate secretion (Simson et al. 1981a).

In these reported studies using acetazolamide in animals, diffusion of bicarbonate from the blood to the lumen was not prevented since the duodenal lumen was perfused with isotonic NaCl which contained no bicarbonate, making possible passive diffusion of bicarbonate.

Recently, it was reported that inhibition of carbonic anhydrase activity by acetazolamide did not alter bicarbonate secretion in the normal human stomach (Feldman & Goldschmiedt 1991), but reduced active bicarbonate transport in the human duodenum when the luminal perfusate contained 24 mmol/l $NaHCO_3$ (Knutson et al. 1992).

Given the likelihood that carbonic anhydrase would serve as a final common pathway in bicarbonate secretion in the duodenum, it was elected to study in detail its actions in the basal and stimulated states of bicarbonate production.

Approach to these issues:

The final part of this thesis was planned to examine the effect of acetazolamide-inhibition of carbonic anhydrase on both basal and stimulated bicarbonate secretion as well as to locate the site of activity of carbonic anhydrase in the guinea pig duodenal crypt and villus structures. Again, emphasis was laid on the cholinergic, VIP and PGE_2 pathways, but several other agonists were also examined in consideration of the concept of "final common pathway."

MATERIALS AND METHODS IN DETAIL

1. GUINEA-PIG MODEL: PERFUSED DUODENAL LOOP WITH ACTIVE SECRETION OF BICARBONATE

Surgical Procedures:

Studies were performed in male albino guinea-pigs (250-500 g, Weizman Institute, Rehovot, Israel). These were kept in a temperature (22°C) and light-controlled (12 hour day/12 hour night) room and fed regular animal pelleted chow ad libitum. They were allowed to adjust to their new environment for at least 4 days prior to experimentation. They were deprived of food for 16-20 hours before the experiments, but were allowed free access to tap water up to the beginning of the experiments.

Animals were anaesthetized with injection of urethane (1.5 g/kg, dissolved in 154 mmol/l sterile NaCl) intra-peritoneally, followed immediately by an injection of sodium indomethacin trihydrate (5 mg/kg, dissolved in 154 mmol/l sterile NaCl) intraperitoneally to inhibit endogenous prostaglandin synthesis (Flemstrom & Garner 1982). Body temperature was maintained at 37°C to 37.5^{o}C by use of a heated blanket and lamp and controlled automatically by an intrarectal thermometer (Harvard Homeothermic Blanket

Control Unit, Harvard Inc., South Natick, MA, USA). A tracheostomy was performed and the animals breathed humidified room air spontaneously.

One (occasionally two) external jugular vein was cannulated (polyethylene tubing I.D. 0.58 mm, Portex, Kent, England) for administration of a physiological solution (2 ml/h, Harvard Apparatus Syringe Infusion Pump Model 22, Harvard Inc., South Natick, MA, USA) containing (in mmol/l) Na^+ 145, K^+ 2.5, Ca^{2+} 0.75, HCO_3^- 24, Cl^- 124 and glucose 5.5, and for injection of drugs as required.

The abdominal cavity was opened by a midline incision, and the peritoneal cavity was rinsed with warm 154 mmol/l sterile NaCl to remove excess urethane. A 2 cm long loop of proximal duodenum was then fashioned between the bile and pancreatic ducts (which enter the duodenum separately in this species). A polyethylene catheter (I.D. 1.14 mm, Portex, Kent, England) was inserted into the duodenum just distal to the entry of the bile duct, and a ligature tied at this point; a second catheter exited the duodenum just proximal to the entry of the pancreatic duct, where another ligature was applied. Great care was taken not to disturb the vascular-neural supply of the duodenum or the free drainage of gastric and biliary secretions (via catheters inserted through the stomach wall) and pancreatic secretions (into the distal duodenum).

The abdomen and neck were closed lightly and kept moist with warm 154 mmol/l NaCl.

General Design of the Studies:

The duodenal segment was perfused (0.5 ml/min, Harvard Apparatus Syringe Infusion Pump Model 22, Harvard Inc., South Natick, MA, USA) with a solution (37°C) containing $NaHCO_3$ 24 mmol/l, with NaCl 130 mmol/l added for isotonicity to 290 mOsmol/kg (Odes et al. 1990). Since the blood bicarbonate concentration in our anaesthetized guinea-pigs is verified by measurement to be 21 $\pm$ 1 mmol/l, this ensured that the duodenum was secreting bicarbonate into the lumen against a concentration gradient and not by diffusion.

The duodenal perfusate solution was pre-gassed with 95% O_2 and 5% CO_2 to bring its pH down from 8.22 to 7.32, so that the infused solution entered the duodenum at a pH similar to plasma; gassing the solution with N_2, as in the back-titration method, rapidly returned its pH to the original value (Odes et al. 1990).

After the resting bicarbonate output had become stable (usually by 20 min from the commencement of perfusion), the basal bicarbonate output was measured for a fixed period of 40 min.

Determination of Bicarbonate Output:

The effluent from the duodenum was collected by continuous gravity drainage into glass measuring cylinders

in 10 min aliquots. The continuous drainage ensured that the duodenal segment never became distended, which might induce peristalsis, or that perfusate was retained. The 10 min volumes were carefully monitored to confirm that the recovered volumes exceeded the infused volumes (usually by 0.2 to 0.5 ml/10 min). Volumes were read to the nearest 0.1 ml and the bicarbonate content was measured immediately by back-titration (Odes et al. 1990). Duplicate, well-mixed 0.5 ml samples of the effluent were acidified with 0.25 ml H_2SO_4 (100 mmol/l), dissolved in 5 ml of CO_2 free H_2O, gassed with N_2 washed in $Ba(OH)_2$, and titrated in glass cups, under continuous N_2 gassing, with NaOH (15 mmol/l) to end-point pH 8.40 using automatic titration apparatus (pH Meter 82, Titrator 80, Autoburette 80, Titration Station 80, all from Radiometer, Copenhagen, Denmark).

In each set of experiments, the concentration of NaOH was verified by titration to end-point pH 7.00 with 100 mmol/l HCl. The pH meter was calibrated daily with buffers of pH 7.00 and 10.00 (Radiometer, Copenhagen, Denmark). Standards of $NaHCO_3$ (24, 10 and 2.5 mmol/l) were run daily.

On randomly selected animals, pancreatic amylase was measured, by starch digestion method, to exclude the presence of aberrant pancreatic ducts in the segment. Amylase was not found.

Monitoring Procedures:

Three procedures were used to monitor the health of the guinea-pig in these studies.

The internal carotid artery was routinely cannulated and blood pressure and pulse were monitored continuously on a Gilson Polygraph (Gilson Medical Electronics, Middleton, WI).

The integrity of the perfused duodenal loop was monitored using Multistix[R] (Ames, Stoke Poges, Slough, UK) to detect any blood, protein and glucose in the duodenal effluent.

Also, duodenal segments were removed immediately at the conclusion of randomly selected experiments and placed in formalin for examination by light microscopy using haematoxylin and eosin staining.

Any animals developing hypotension were not included; this was not common (2% of studies) and occurred where blood loss was excessive during the surgery, or where hexamethonium was infused i.v. Any evidence of damage to the duodenal loop meant that the experiment was discarded. Such events were not common.

Calculations:

The amount of bicarbonate perfused through the duodenal lumen per 10 min period was subtracted from the recovered bicarbonate to yield the net secreted bicarbonate. The output of bicarbonate was expressed as $\mu mol.cm^{-1}.10\ min^{-1}$. Thirty min basal bicarbonate outputs were obtained by summing the three 10 min outputs obtained prior to the first injection of any test agent, and 30 min "stimulated" bicarbonate outputs by summing the first three 10 min-periods following injection of a drug or combination of two drugs. In the series of experiments using the VIP-antagonist, the 30 min bicarbonate outputs represented serial test periods.

2. DETERMINATION OF ADENYLATE CYCLASE AND PROTEIN KINASE A IN ISOLATED DUODENAL ENTEROCYTES

Male albino guinea-pigs were killed by a blow on the head and a 5 cm segment of the duodenum distal to the entry of the bile duct was resected immediately.

Duodenal epithelial cells were isolated essentially according to the method of Weiser (1973). The lumen of the resected duodenum was flushed with ice-cold, oxygenated

saline. One end was clamped with a hemostat and the lumen filled with a buffer containing (in mmol/l) NaCl 96, KCl 1.5, Na-citrate 27, K_2HPO_4 2.8, KH_2PO_4 2.8, DTT 0.5; at pH 7.3. After 15 min of incubation at 37°C with continuous O_2 gassing the solution was discarded and the duodenum refilled with a solution containing (in mmol/L) NaCl 140, Na_2HPO_4 16, EDTA 1.5, DTT 0.5; at pH 7.3. The duodenum was incubated twice for 15 min at 37°C under continuous gassing in this buffer. The released cells were washed twice in HEPES-buffered saline containing (in mmol/l): NaCl 70, KCl 5, NaH_2PO_4 0.5, Na_2HPO_4 1, $NaHCO_3$ 20, HEPES 25, at pH 7.4, then resuspended in homogenization buffer (in mmol/L: TRIS-HCl 25, EDTA 0.1, DTT 0.1; at pH 7.8) and sonicated (Branson Instruments; 30 W, 3 x 5 sec).

Adenylate cyclase activity in homogenates of the duodenal cells was determined by a competitive protein binding assay, based on Schwabe et al. (1974) as described by us (Reimer et al. 1992). Isolated cells (0.6 - 0.8 mg of protein per ml) were incubated in MEM supplemented with 1% bovine serum albumin (BSA, pH 7.4) at 37°C for the indicated periods of time under continuous gassing with 95% O_2/5% CO_2. The incubation was terminated by heating aliquots of the cells to 95°C for 5 min. Subsequently, the denatured protein was pelleted at 10,000 g. Cyclic AMP content in the supernatant was determined by radioimmunoassay, as described previously by us. Standards were prepared in the incubation

medium and treated as the samples. Adenylate cyclase activity was expressed as pmol cAMP.mg $protein^{-1}.min^{-1}$.

Protein kinase assay: Isolated cells (1.5 - 2.0 mg of protein per ml) were incubated at 37°C for 30 min under the same conditions as described above, except that 0.1 mmol/l IBMX was included, as indicated. Incubations were stopped by cooling the cells on ice and washing them once in ice-cold, BSA-free incubation buffer containing 0.5 mmol/l IBMX. The cells were disrupted by sonication in 1 ml homogenization buffer containing (mmol/l): TRIS/HCl 20 , sucrose 250, EDTA 2, EGTA 1, NaCl 150, DTT 10; pH 7.4. For studying the subcellular distribution, the cell homogenate was centrifuged for 30 min at 100,000 x g. The supernatant recovered was used as the cytosolic fraction. The pellets were solubilized by stirring in 1 ml homogenization buffer containing 0.2% Triton-X 100 and used as a crude membrane preparation. Mg^{2+}-dependent protein kinase activity was assayed by measuring the incorporation of $^{32}PO_4{}^{3-}$ from ATP into histone in an assay mixture (0.2 ml) containing (mmol/l): Pipes/TRIS 10, EGTA 0.2, $MgCl_2$ 2, NaF 5, IBMX 0.1; pH 6.8 and ^{32}P-ATP 40 μmol/l (200-300 cpm/pmol), 50 μg histone (type II-A). To determine the total protein kinase A activity, 1 μmol/l cAMP was included in the incubation medium, when indicated. The reaction was started by the addition of 20 μl of enzyme preparation. Phosphorylation reaction was measured with 10 μg of enzyme preparation for 5 min at 30°C. Under these conditions, the reaction rates were

linear with time. The reaction was stopped by the addition of 0.2 ml 10 % trichloroacetic acid (TCA) containing 5 mmol/l unlabelled ATP and 15 mmol/l K_2HPO_4. The acid precipitable material was collected on Millipore membrane filters (pore size: 0.45 μm) and washed twice with 10 ml 5 % TCA. The incorporation of $^{32}PO_4{}^{3-}$ into histone was measured by scintillation counting.

3. DESIGN OF EXPERIMENTS IN THE GUINEA-PIG IN VIVO DUODENAL SEGMENT

General note: To exclude any "placebo effects", the vehicles of all agents used were injected separately after the basal period of bicarbonate secretion; no effects on bicarbonate secretion were detected, and these data were omitted from the Results.

3.1. STIMULATORY ROLE OF PEPTIDE HORMONES, PROSTAGLANDINS AND CYCLIC NUCLEOTIDES ON BICARBONATE SECRETION

Dose Response Studies:

Basal bicarbonate output was measured for a period of 40 min, as was done in all the experiments, and it was established that basal secretion was quite steady. It should be noted that the basal secretion rate does vary in relationship to animal weight, and this affects the peak response when agonists are administered. However, I prefer to show the exact values obtained in the experiments, rather than values which are "corrected for weight" that tend to distort the data.

The structurally related peptides: VIP, glucagon and secretin were infused i.v. in increasing doses through the range 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} $mol.kg^{-1}.30\ min^{-1}$, and the bicarbonate output measured. This was done to enable a comparison of their potencies to be made.

The effect of secretin on pancreatic bicarbonate secretion was also determined. Secretin was infused as described, and the pancreatic juice entering the duodenum was collected, diluted, and submitted immediately to back-titration for measurement of bicarbonate secretion.

In a separate experiment, TPA (10^{-7} mol/kg) was infused over 30 min and the effect on bicarbonate secretion

measured; this agent was selected since it acts in cells directly via protein kinase C.

The VIP-antagonist was infused (3.3 $mg.kg^{-1}$, equal to 10^{-6} mol/kg) together with VIP in another experiment to determine the specificity of the VIP-stimulation.

In another study, the potency of VIP, dBcAMP, PGE_2 and $PGF_{2\alpha}$ was compared. Since VIP is thought to act via cAMP (Smith 1990), dBcAMP and PGE_2 were tested, while $PGF_{2\alpha}$ was selected for comparison because its mode of action is different. VIP, PGE_2 and $PGF_{2\alpha}$ were given i.v., while dBcAMP was administered by intraduodenal infusion (since we had determined that dBcAMP is more potent by this route compared with the i.v. route). All agents were infused in increasing doses (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} $mol.kg^{-1}.30\ min^{-1}$), each dose being given for 30 min.

Then, dBcAMP, dBcGMP and theophylline were tested for their relative potency in stimulating active secretion of bicarbonate. All agents were infused i.v. in increasing doses (10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} $mol.kg^{-1}.30\ min^{-1}$), with each dose being administered over 30 min.

Combinations of Agonists:

The aforementioned experiments allowed a decision to be made as to which agonists were the most potent and suggested that both the adenylate cyclase/protein kinase A cascade and the inositol phospholipid/protein kinase C cascade participated in bicarbonate secretion. Experiments were then designed where two agonists were infused together to see whether additive effects on bicarbonate secretion would occur.

The combined infusion of glucagon with VIP will illustrate the design of these experiments. Glucagon (10^{-6} $mol.kg^{-1}.30\ min^{-1}$) was infused, after the basal period, for 90 min, and VIP (10^{-11} $mol.kg^{-1}.30\ min^{-1}$) was infused in the middle 30 min period of the glucagon infusion. Therefore, glucagon was infused alone before and after the combined glucagon and VIP infusion. The effect of glucagon was to stimulate bicarbonate secretion. The question was, whether VIP would further increase the bicarbonate secretion. It will be noted that glucagon was given in a dose that cannot produce maximal bicarbonate secretion; in other words, the glucagon dose was submaximal. The VIP dose was relatively low, but able to stimulate bicarbonate secretion.

Similarly experiments were performed on the following combinations:

glucagon (10^{-6} mol.kg^{-1}.30 min^{-1}) and PGE_2 (10^{-7} mol.kg^{-1}.30 min^{-1});

glucagon (10^{-6} mol.kg^{-1}.30 min^{-1}) and $PGF_{2\alpha}$ (10^{-6} mol.kg^{-1}.30 min^{-1});

VIP (10^{-11} mol.kg^{-1}.30 min^{-1}) and PGE_2 (10^{-7} mol.kg^{-1}.30 min^{-1});

VIP (10^{-11} mol.kg^{-1}.30 min^{-1}) and $PGF_{2\alpha}$ (10^{-6} mol.kg^{-1}.30 min^{-1});

PGE_2 (10^{-7} mol.kg^{-1}.30 min^{-1}) and $PGF_{2\alpha}$ (10^{-6} mol.kg^{-1}.30 min^{-1});

dBcAMP (5×10^{-8} mol.kg^{-1}) and PGE_2 (5×10^{-7} mol.kg^{-1});

dBcAMP (5×10^{-8} mol.kg^{-1}) and VIP (5×10^{-9} mol.kg^{-1}).

In all cases the doses used were submaximal and the objective was to see whether the effects on duodenal bicarbonate secretion were additive.

Detection of Intracellular Messengers:

These related studies were performed at the Medical School, Hannover, Germany (Dr Reimer). The effect of PGE_2 and VIP on the generation of cAMP and protein kinase activity in homogenates of isolated duodenal enterocytes was measured.

3.2. CHOLINERGIC STIMULATION OF BICARBONATE SECRETION

Effects on Basal Bicarbonate Secretion of Selected Cholinergic Agonists and Antagonists:

After basal bicarbonate secretion was measured, injections of the following agonists were administered:
(1) carbachol (in the doses 0.2 $\mu g.kg^{-1}.5\ min^{-1}$, which equals 10^{-9} $mol.kg^{-1}.5\ min^{-1}$; and 2 $\mu g.kg^{-1}.5\ min^{-1}$, which equals 10^{-8} $mol.kg^{-1}.5\ min^{-1}$);
(2) calcium ionophore A23187 (5, 50 and 500 $\mu g.kg^{-1}.5\ min^{-1}$, corresponding to 10^{-8}, 10^{-7} and 10^{-6} $mol.kg^{-1}.5\ min^{-1}$).

These agents were infused i.v. in a volume of 0.5 ml over 5 minutes to determine their effects on basal bicarbonate secretion. The increasing doses of carbachol and A23187, as specified, were used to obtain dose-response data for these drugs; these doses were administered in random order at 40 min intervals.

The following agents were tested for their inhibitory effect on basal bicarbonate output.
(1) atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$, corresponding to 1.5 x 10^{-7} $mol.kg^{-1}.5\ min^{-1}$);
(2) pirenzepine (1 $mg.kg^{-1}.5\ min^{-1}$, corresponding to 2 x 10^{-6} $mol.kg^{-1}.5\ min^{-1}$);
(3) hexamethonium (0.3 $mg.kg^{-1}.5\ min^{-1}$, corresponding to 10^{-6} $mol.kg^{-1}.5\ min^{-1}$);

(4) verapamil (0.2 $mg.kg^{-1}.5\ min^{-1}$, which corresponds to 4 x 10^{-7} $mol.kg^{-1}.5\ min^{-1}$).

These agents were also infused i.v. in a volume of 0.5 ml over 5 minutes to determine their effects on basal bicarbonate secretion.

By the end of 30 min after any of these treatments, the bicarbonate output had returned to basal.

Effect of Inhibition of Carbachol-Stimulated Bicarbonate Secretion:

In this series of experiments, the effect on carbachol-stimulated bicarbonate secretion was tested of the anticholinergics: atropine, pirenzepine and hexamethonium, and the Ca^{2+} channel blockers verapamil and nifedipine. After basal bicarbonate secretion was determined, carbachol (2 $\mu g.kg^{-1}.5\ min^{-1}$, corresponding to 10^{-8} $mol.kg^{-1}.5\ min^{-1}$) was injected i.v. on three occasions every 40 min; atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$), pirenzepine (1 $mg.kg^{-1}.5\ min^{-1}$), hexamethonium (0.3 $mg.kg^{-1}.5\ min^{-1}$), verapamil (0.2 $mg.kg^{-1}.5\ min^{-1}$) or nifedipine (1.7 $mg.kg^{-1}.5\ min^{-1}$, corresponding to 5 x 10^{-6} $mol.kg^{-1}.5\ min^{-1}$) was injected i.v. 5 minutes prior to the second administration of carbachol.

Role of VIP in the Cholinergic Stimulation of Duodenal Bicarbonate Secretion:

The role of VIP in the cholinergic pathway of duodenal bicarbonate secretion was examined using VIP and the VIP-antagonist [D-p-Cl-Phe^{6},Leu^{17}]-VIP.

The effect of the antagonist on basal and VIP-stimulated bicarbonate secretion was examined. After basal bicarbonate output was measured for 30 min, sequential 30-min i.v. infusions were administered of the antagonist alone (3.3 $mg.kg^{-1}$), then the antagonist (3.3 $mg.kg^{-1}$, or 10^{-6} mol/kg) and VIP (5 $\mu g.kg^{-1}$) simultaneously via different veins, and finally VIP (5 $\mu g.kg^{-1}$) alone.

Next, after determination of basal bicarbonate output, the VIP-antagonist (3.3 $mg.kg^{-1}$) and carbachol (4 $\mu g.kg^{-1}$) were infused i.v. simultaneously for 30 min, followed by carbachol (4 $\mu g.kg^{-1}$) alone for another 30 min.

Thirdly, the effect of pirenzepine (1 $mg.kg^{-1}.5\ min^{-1}$) or atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$) on VIP-stimulated bicarbonate secretion was tested by infusing VIP 5 $\mu g.kg^{-1}$ for 3 periods of 30 minutes, while pirenzepine or atropine was injected prior to the second VIP treatment.

The VIP-antagonist was given as a continuous infusion since it has a very short half-life (Algazi et al. 1989). For this reason, VIP, carbachol and A23187 were also infused continuously in these experiments.

In addition, the role of VIP in A23187-stimulation of bicarbonate secretion was also determined, as follows. After stimulation by A23187 was determined, the effect of [D-p-Cl-Phe^{6},Leu^{17}]-VIP on A23187-stimulated bicarbonate secretion

was tested by infusing the VIP-antagonist during stimulation with A23187.

Detection of M-Cholinoceptors on Duodenal Enterocytes; Measurement of Intracellular Ca^{2+} after Cholinergic Stimulation:

The intention was to find out if duodenal enterocytes have muscarinic cholinergic receptors. Basolateral membranes prepared from isolated duodenal enterocytes were tested for their ability to bind ^{3}H-N-methylscopolamine. Intracellular Ca^{2+} levels were determined in isolated intact duodenal enterocytes in the basal state and after stimulation with carbachol and related secretagogues. These experiments were performed on our behalf by Dr. R Reimer and the details of the methodology were recently published (Reimer et al. 1993a).

3.3. ROLE OF SOMATOSTATIN IN DUODENAL BICARBONATE SECRETION

Somatostatin and Basal Bicarbonate Secretion:

In the first series of experiments, basal bicarbonate secretion was determined for 30 minutes, and then somatostatin-14 was infused intravenously in the dose of

10^{-11} mol/kg over 30 minutes; this was followed by further sequential infusions of somatostatin-14 in the doses of 10^{-9} mol/kg and 10^{-7} mol/kg, each for 30 minutes, to determine the effect of this peptide on basal bicarbonate secretion. Then, PGE_2 10^{-6} mol/kg was infused for the final 30 minutes to demonstrate the bicarbonate secretory capacity of the duodenum.

Somatostatin and Stimulated Bicarbonate Secretion:

In the next series of experiments, after basal bicarbonate secretion had been determined, carbachol (10^{-8} mol/kg), VIP (10^{-8} mol/kg) and PGE_2 (10^{-8} mol/kg) were infused i.v. for three consecutive periods of 30 minutes each; somatostatin-14 (10^{-7} mol/kg) was infused during the second 30 minute period of agonist-infusion.

Effect of Somatostatin on cAMP Formation:

Adenylate cyclase activity in homogenized duodenal enterocytes was measured in the basal state as well as in the presence of somatostatin (10^{-6} mol/l), carbachol (10^{-3} mol/l), VIP (10^{-8} mol/l) or PGE_2 (10^{-7} mol/l). The effect of somatostatin in the presence of these agonists was also tested.

3.4. ROLE OF CARBONIC ANHYDRASE ENZYME IN DUODENAL BICARBONATE SECRETION

Dose-Response Effect of Acetazolamide on Basal Bicarbonate Secretion:

The role of carbonic anhydrase in bicarbonate secretion was studied using acetazolamide. After measuring the bicarbonate output under basal conditions for 30 minutes, acetazolamide was infused i.v. as a single dose of 80 $mg.kg^{-1}.5\ min^{-1}$ (which is equal to 3.6×10^{-4} mol/kg), and the effect on bicarbonate secretion was noted.

Effect of Inhibition of Carbonic Anhydrase on Stimulated Bicarbonate Secretion:

The role of carbonic anhydrase in stimulated bicarbonate secretion was also studied. The following agonists, that utilize different intracellular messengers, were infused i.v. for three consecutive periods of 30 minutes each: PGE_2 (10^{-6} $mol.kg^{-1}.30\ min^{-1}$), $PGF_{2\alpha}$ (10^{-6} $mol.kg^{-1}.30\ min^{-1}$), TPA (10^{-7} $mol.kg^{-1}.30\ min^{-1}$), glucagon (10^{-7} $mol.kg^{-1}.30\ min^{-1}$), VIP (10^{-8} $mol.kg^{-1}.30\ min^{-1}$) and carbachol (10^{-8} $mol.kg^{-1}.30$
min^{-1}). Similarly, dBcAMP (10^{-5} $mol.kg^{-1}.30\ min^{-1}$) and dBcGMP (10^{-5} $mol.kg^{-1}.30\ min^{-1}$) were infused intraduodenally. Acetazolamide, in the dose of 80 $mg.kg^{-1}.5$

min^{-1}, was infused by a separate vein during the middle 30 minute period of agonist-stimulation for each of these agents.

Histochemical Localization of Carbonic Anhydrase in the Duodenum of the Guinea-Pig:

A duodenal segment corresponding to the perfusion studies in vivo was removed and quickly rinsed in isotonic saline. Pieces of 1 cm were immediately frozen in liquid nitrogen. From these specimens, 10 µm sections were prepared in a cryostat at -25°C and mounted on glass slides. For the demonstration of carbonic anhydrase according to Dermietzel et al. (1985), the sections were incubated with 100 µl of 5.10^{-5} mol/l dimethylamino-naphthaline-5-sulfonamide (DNSA) in phosphate buffered saline for 10 min at room temperature. For counterstaining of nuclei, the sections were incubated additionally with propidium iodide (5 µg/ml) for 10 sec. Control sections of duodenum were incubated with DNSA in the presence of the nonfluorescent inhibitor acetazolamide (1 mmol/l). Sections of guinea pig stomach, known to be rich in carbonic anhydrase, were incubated with DNSA for a positive control. The blue fluorescence of the carbonic anhydrase-DNSA complex was visualized and photographed using a fluorescence microscope (Olympus, Germany) at an excitation wavelength of 470 nm.

4. SOURCES OF REAGENTS

All drugs and chemicals were purchased from Sigma, St Louis, MO, except indomethacin which was a gift from Merck Sharp & Dohme, Rahway, NJ, USA, and the synthetic prostaglandins E_2 and F_{2a} which were kind gifts from the Upjohn Co., Kalamazoo, MI, USA.

All drugs were freshly prepared on the days of experiment. Most drugs were dissolved in 154 mmol/l NaCl.

VIP was dissolved in 154 mmol/l NaCl which also contained 1 mmol/l ascorbic acid. The VIP-antagonist [D-p-Cl-Phe^6,Leu^{17}]-VIP which was dissolved in 154 mmol/l NaCl with 0.1% bovine serum albumin and 1 mmol/l ascorbic acid added. Glucagon was dissolved in 154 mmol/l NaCl containing 0.5 mg/ml bovine serum albumin and 15 mmol/l NaOH at pH 8.9, and the pH was then adjusted to 7.4 with 100 mmol/l HCl. The prostaglandins were dissolved in a few drops of absolute ethyl alcohol and diluted in 100 mmol/l phosphate buffer. The Ca^{2+} ionophore A23187 was dissolved in dimethyl sulfoxide (DMSO).

5. STATISTICAL ANALYSIS

Results are shown as mean ± SEM for at least 4 animals or preparations, unless indicated otherwise. Each animal served as its own control. Student's paired t-test (and sometimes unpaired test) and analysis of variance were used to compute the statistical significance of differences, and P values < 0.05 were considered to be significant. Where multiple P values of varying degrees of significance were obtained, and it was felt that printing each specific P value would be burdensome to the reader and not add to the meaning of the observations, the general term "P at least > 0.05" was employed.

RESULTS

VALIDATION OF THIS MODEL: ACTIVE SECRETION OF BICARBONATE IN VIVO

To test, whether bicarbonate is actively secreted by the guinea-pig duodenum, the lumen was perfused with 24 mmol/l $NaHCO_3$, made isotonic to the body fluid with NaCl. The results show that bicarbonate secretion in the presence of luminal 24 mmol/l $NaHCO_3$ was equal to the secretion obtained during perfusion with 154 mmol/l NaCl alone (Fig. 1).

MONITORING PROCEDURES

The luminally perfused duodenal loops retained their normal colour and configuration during the studies. Histological examination (Fig. 2) of the duodenum showed that the tissues remained healthy and revealed no signs of degeneration at the end of the experiments. Brunner's glands were occasionally evident in the submucosa. Blood, protein and glucose were absent from the duodenal effluent. The blood pressure (130/90 mm Hg) and pulse rate were stable and within the normal range with all the drugs tested in the doses indicated, except hexamethonium, which induced severe

hypotension (systolic pressure 50 mm Hg) and bradycardia immediately when injected, with slow recovery to normal over 60 min.

It should be noted that higher doses of some of the tested agents adversely affected the blood pressure, preventing us from measuring a saturation kinetic for these agonists.

STIMULATION OF DUODENAL BICARBONATE SECRETION BY PEPTIDE HORMONES, PROSTAGLANDINS AND CYCLIC NUCLEOTIDES

Dose-Response Interrelationships of Agonists of Bicarbonate Secretion:

Basal bicarbonate secretion varied in the range of 8 to 29 μmol.cm^{-1}.30 min^{-1}, depending on animal weight.

VIP increased duodenal bicarbonate secretion significantly in a dose-response manner (Fig. 3). The effect of the VIP-antagonist [D-p-Cl-Phe^{6},Leu^{17}]-VIP on basal and VIP-stimulated bicarbonate secretion was studied. Duodenal bicarbonate secretion was 10.5 $\pm$ 0.5 μmol.cm^{-1}.30 min^{-1} under basal conditions, 10.6 $\pm$ 1.1 μmol.cm^{-1}.30 min^{-1} during

FIGURE 1. Basal bicarbonate secretion in the isolated guinea-pig proximal duodenum perfused with 154 mmol/l NaCl or 24 mmol/l $NaHCO_3$ (plus 130 mmol/l NaCl for isotonicity). The bicarbonate secretion in the presence of luminal $NaHCO_3$ was equal to the secretion obtained during perfusion with NaCl alone. n = 6.

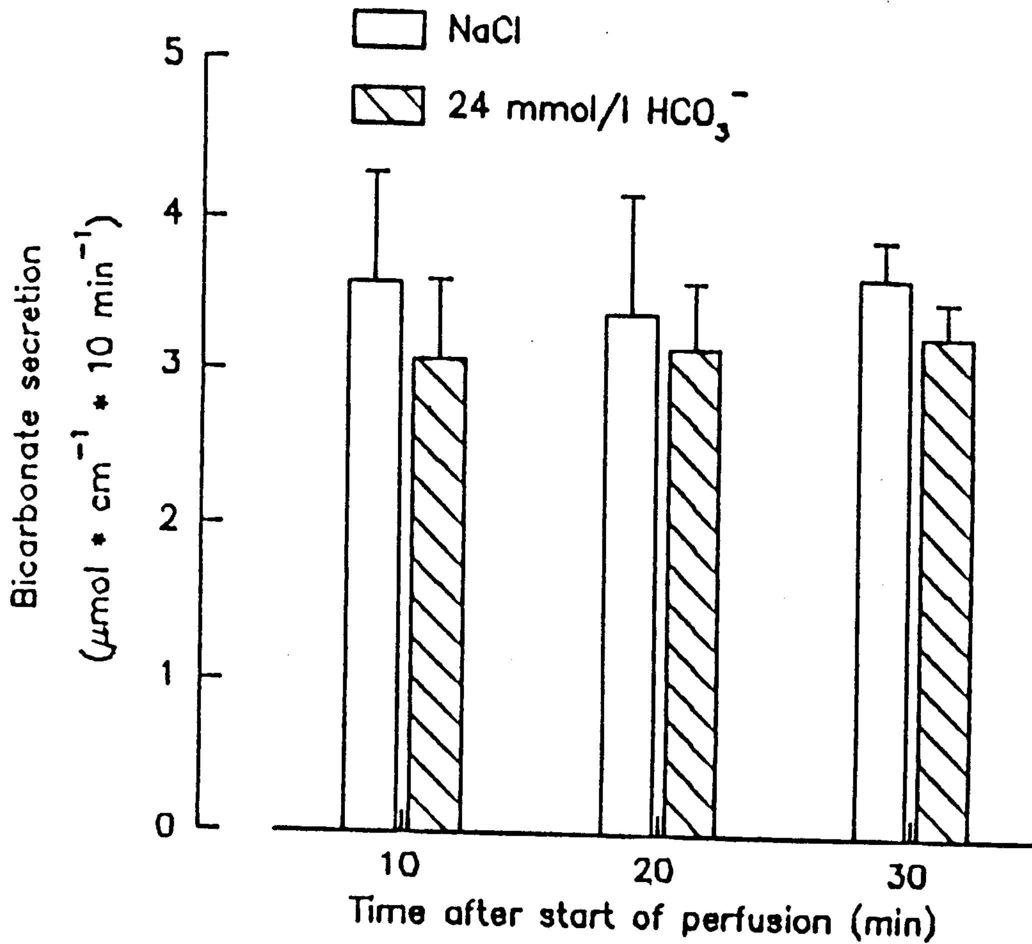


FIGURE 2. Histology of isolated guinea-pig duodenal segment at the conclusion of a typical experiment. The tissue is well preserved and no signs of degeneration are evident. Very small numbers of Brunner's glands are seen in the submucosa; their contribution to bicarbonate secretion is regarded as slight (see Text).

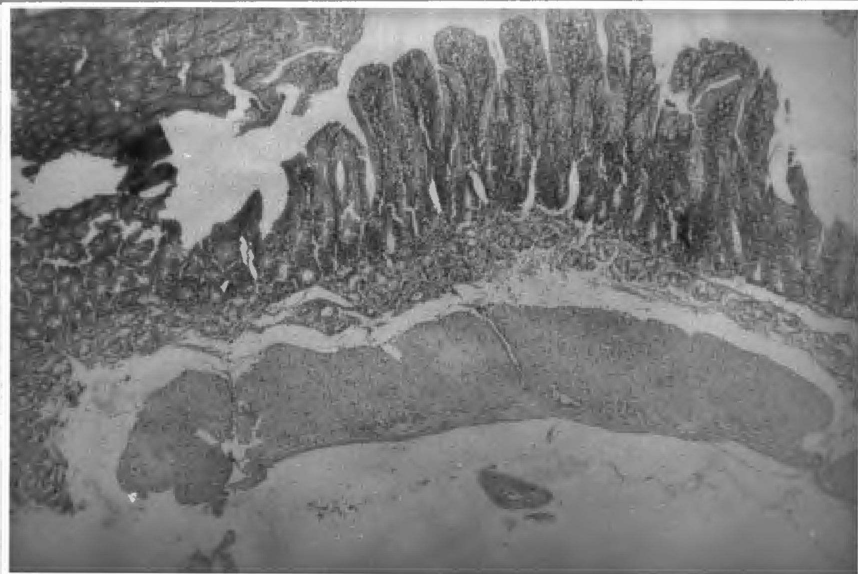
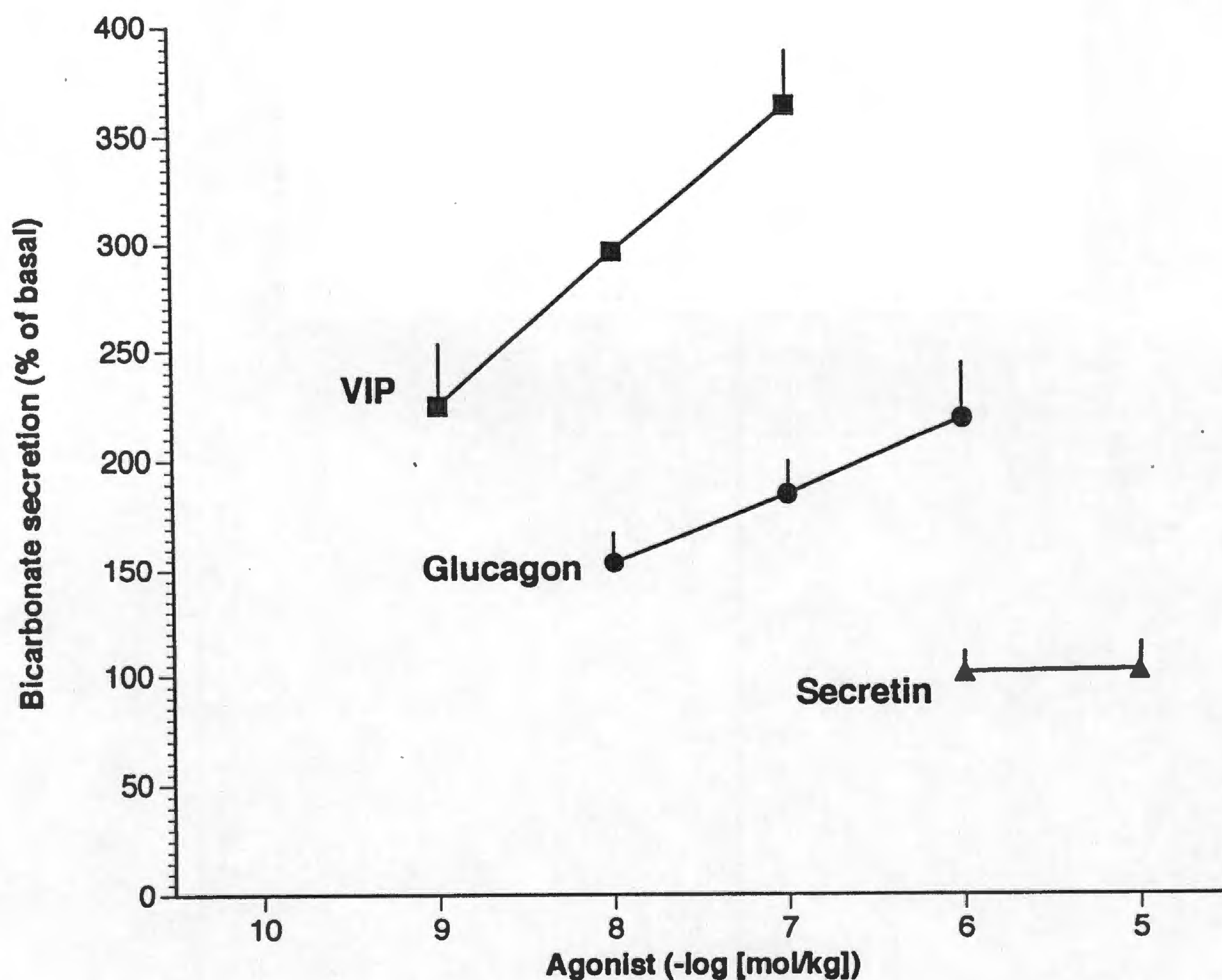


FIGURE 3. Concentration response relationship of selected peptide hormones for stimulation of duodenal bicarbonate secretion. Basal bicarbonate secretion was the following: VIP 8.6 ± 1.1 $\mu mol.cm^{-1}.30\ min^{-1}$, glucagon 9.9 ± 0.7 $\mu mol.cm^{-1}.30\ min^{-1}$ and secretin 9.5 ± 0.6 $\mu mol.cm^{-1}.30\ min^{-1}$. All agents were infused i.v. Values are mean ± SEM from 4 to 8 animals. P at least < 0.05 for differences from basal and between all doses for VIP and for glucagon. Secretin did not stimulate duodenal bicarbonate secretion.



infusion of [D-p-Cl-Phe^6,Leu^{17}]-VIP (10^{-6} mol.kg^{-1}, or 3.3 mg.kg^{-1}) alone, and 12.7 ± 1.0 μmol.cm^{-1}.30 min^{-1} (P = NS) when the VIP-antagonist and VIP (5 μg.kg^{-1}) were infused simultaneously. VIP alone stimulated the bicarbonate secretion to 21.1 ± 1.5 μmol.cm^{-1}.30 min^{-1} ($P < 0.01$). The effect of VIP was therefore completely inhibited by the VIP-antagonist [D-p-Cl-Phe^6,Leu^{17}]-VIP in the dose 3.3 mg.kg^{-1}.

Glucagon increased duodenal bicarbonate secretion in a dose-dependant fashion (Fig. 3). VIP was more potent than glucagon on a molar basis ($P < 0.005$). Secretin, given at even higher doses than glucagon, had no effect on the duodenal bicarbonate secretion (Fig. 3); in this dose range, secretin however increased pancreatic bicarbonate output from 282 to 1473 μmol.30 min^{-1}.

In another experiment, VIP (given i.v.), PGE_2 (given i.v.) and dBcAMP (given intraduodenally) were shown to stimulate bicarbonate secretion significantly up to 400% of the basal output in a dose-dependent manner (Fig. 4). Intraduodenal application of dBcAMP was 30% more effective in stimulation of bicarbonate secretion than i.v. application of this agonist. Furthermore, dBcAMP was arithmetically more potent than VIP, which was somewhat more potent than PGE_2, but these differences were not statistically significant (Table 2).

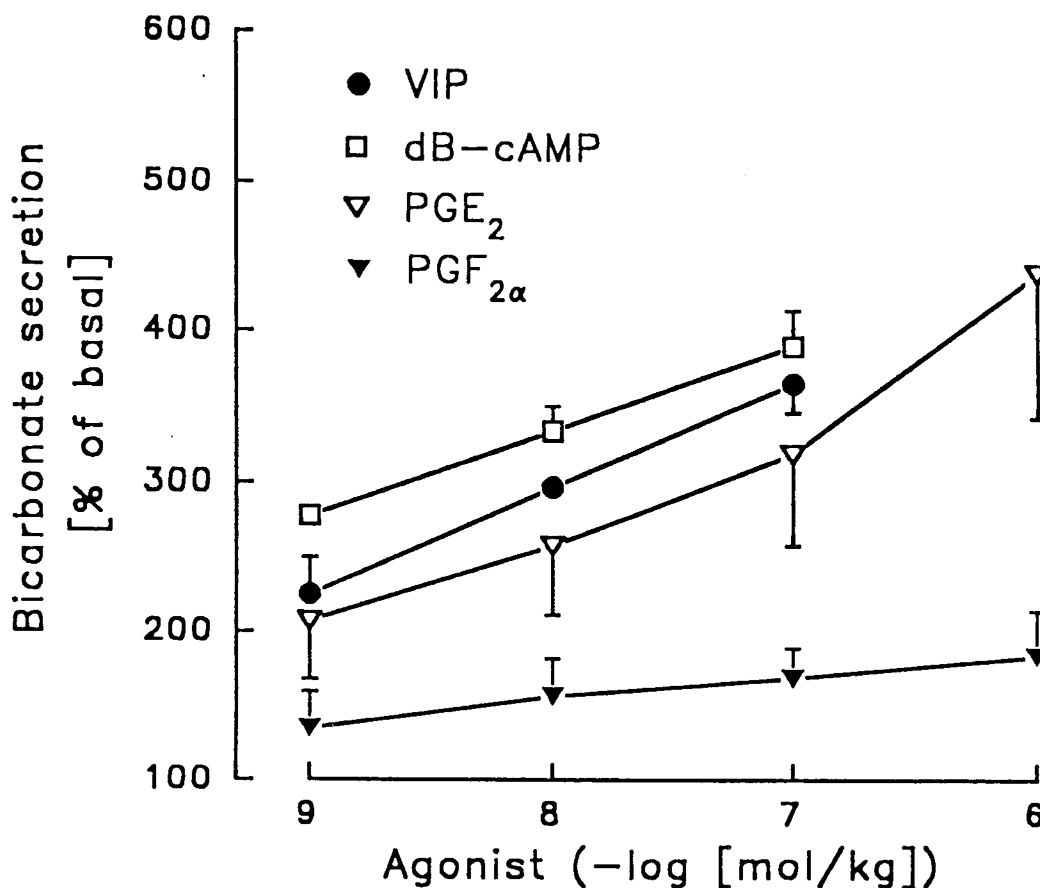
TABLE 2. Rank order of potency of the agonists of duodenal bicarbonate secretion.

Agonist (10^{-8} mol/kg)	HCO_3^- Secretion (% of basal)
dBcAMP *	338 ± 14 [a]
VIP	297 ± 10 [b]
PGE_2	273 ± 37 [c]
Carbachol	260 ± 15 [a,d]
Theophylline	219 ± 25 [a,b,c,e]
dBcGMP	181 ± 14 [a,b,c,d]
PGF_{2a}	169 ± 14 [a,b,c,d]
Glucagon	154 ± 11 [a,b,c,d,e]

Basal bicarbonate secretion was 100%. Each agonist was infused i.v. for 30 minutes. There was no statistically significant difference between dBcAMP, VIP and PGE_2. Agonists with shared superscripts showed significant differences (P at least < 0.05; n at least 4). In addition, TPA (10^{-7} mol/kg) increased basal bicarbonate secretion by 274 ± 14% ($P < 0.05$).

* Note that dBcAMP was given intraduodenally and the other agents i.v.; the comparison with dBcAMP may therefore <u>not</u> be valid.

FIGURE 4. Concentration response relationship of selected agonists for stimulation of duodenal bicarbonate secretion. Basal bicarbonate secretion in these experiments was as follows: VIP 12.0 ± 0.9 μmol.cm^{-1}.30 min^{-1}, PGE_2 17.1 ± 2.0 μmol.cm^{-1}.30 min^{-1}, $PGF_{2\alpha}$ 16.0 ± 0.6 μmol.cm^{-1}.30 min^{-1} and dBcAMP 13.2 ± 0.5 μmol.cm^{-1}.30 min^{-1}. VIP, PGE_2 and $PGF_{2\alpha}$ were infused i.v. and dBcAMP was given intraduodenally. Values are mean ± SEM from 4 to 6 animals. P at least < 0.05 for differences from basal and between doses for all the agonists tested.



Infusion of PGF_{2a} also increased duodenal bicarbonate secretion, although this effect was very small compared to all the other agonists (Fig. 4). PGF_{2a}, at the dose of 10^{-6} mol/kg, increased bicarbonate secretion to 80% above the basal value. PGF_{2a} was much less potent than PGE_2 ($P < 0.005$, Table 2).

The agonists dBcAMP, theophylline and dBcGMP all increased duodenal bicarbonate secretion (Fig. 5). The most potent agonist was dBcAMP; dBcGMP was about one-third as potent as dBcAMP at equivalent molar doses ($P < 0.001$).

Carbachol increased duodenal bicarbonate secretion as follows: when basal bicarbonate secretion was 100%, carbachol 10^{-9} mol/kg stimulated the secretion to 211 ± 15% and carbachol 10^{-8} mol/kg stimulated the secretion to 260 ± 14% ($n = 5$, $P <$ at least 0.05). (These data are considered more fully below and in Fig. 8).

Comparing the molar potency of all the agonists tested in this section, there were arithmetic but not statistical differences between dBcAMP, VIP and PGE_2 (Table 2). Carbachol was less potent than dBcAMP ($P < 0.005$), but did not differ from VIP or PGE_2. Theophylline was less potent than dBcAMP, VIP and PGE_2 ($P < 0.05$). dBcGMP, PGF_{2a} and glucagon were all of lower potency. As noted above, secretin was not an agonist of duodenal bicarbonate secretion.

Relationship between Intracellular cAMP, Protein Kinase A and Bicarbonate Production:

Data produced in the in vivo experiments in the guinea-pig were combined with the in vitro data obtained from isolated guinea-pig duodenal enterocytes (performed by Dr. Reimer, in order to establish the temporal relationship between intracellular cAMP levels, PKA activity and bicarbonate secretion.

Fig. 6 shows the temporal relationship between intracellular cAMP levels, PKA activity and bicarbonate secretion in the guinea-pig treated with VIP.

Isolated duodenal cells were incubated in the presence of 10^{-7} mol/l VIP in the absence of the phosphodiesterase inhibitor IBMX. Intracellular cAMP levels were maximal at 5 min of incubation. PKA activity, given as the ratio of activity assayed in the absence and presence of 1 µmol/l cAMP, reached a maximum after 15 min of incubation. Duodenal bicarbonate secretion, stimulated by i.v. infusion of VIP in the dose 10^{-10} mol/kg, was maximal at 30 min. These data fit together nicely to show that VIP stimulation leads to the generation of cAMP in the duodenal enterocytes, followed by activation of protein kinase A, and finally by secretion of bicarbonate into the duodenal lumen. Exactly the same

FIGURE 5. Concentration response relationship of dBcAMP, dBcGMP and theophylline for stimulation of duodenal bicarbonate secretion. Basal bicarbonate secretion was as follows: dBcAMP 13.2 ± 0.5 μmol.cm^{-1}.30 min^{-1}, dBcGMP 8.2 ± 0.4 μmol.cm^{-1}.30 min^{-1} and theophylline 7.9 ± 0.2 μmol. cm^{-1}.30 min^{-1}. The agonists were injected i.v. Values are mean ± SEM from 6 animals. P at least < 0.05 for differences from basal and between doses for all agonists tested.

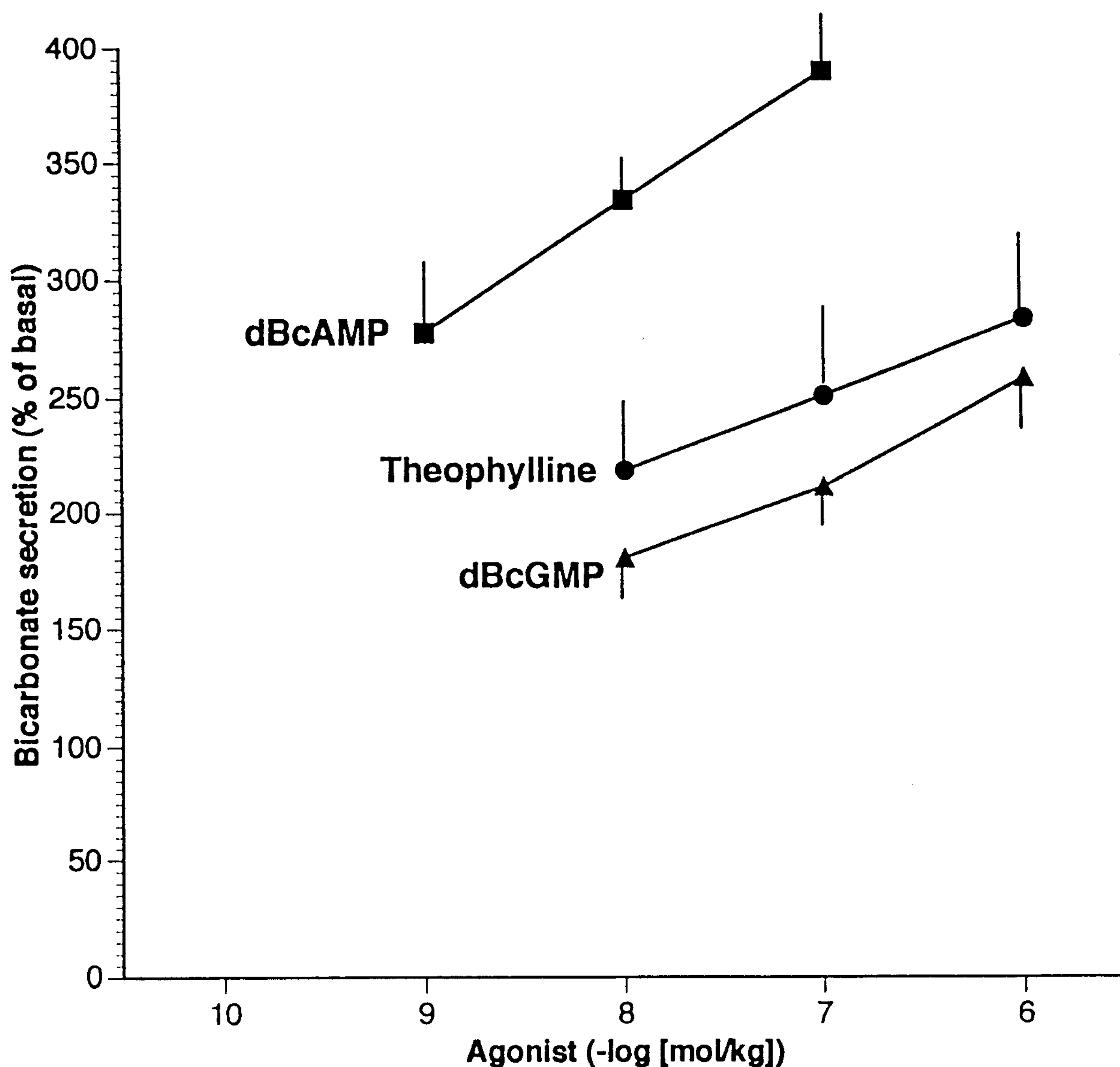
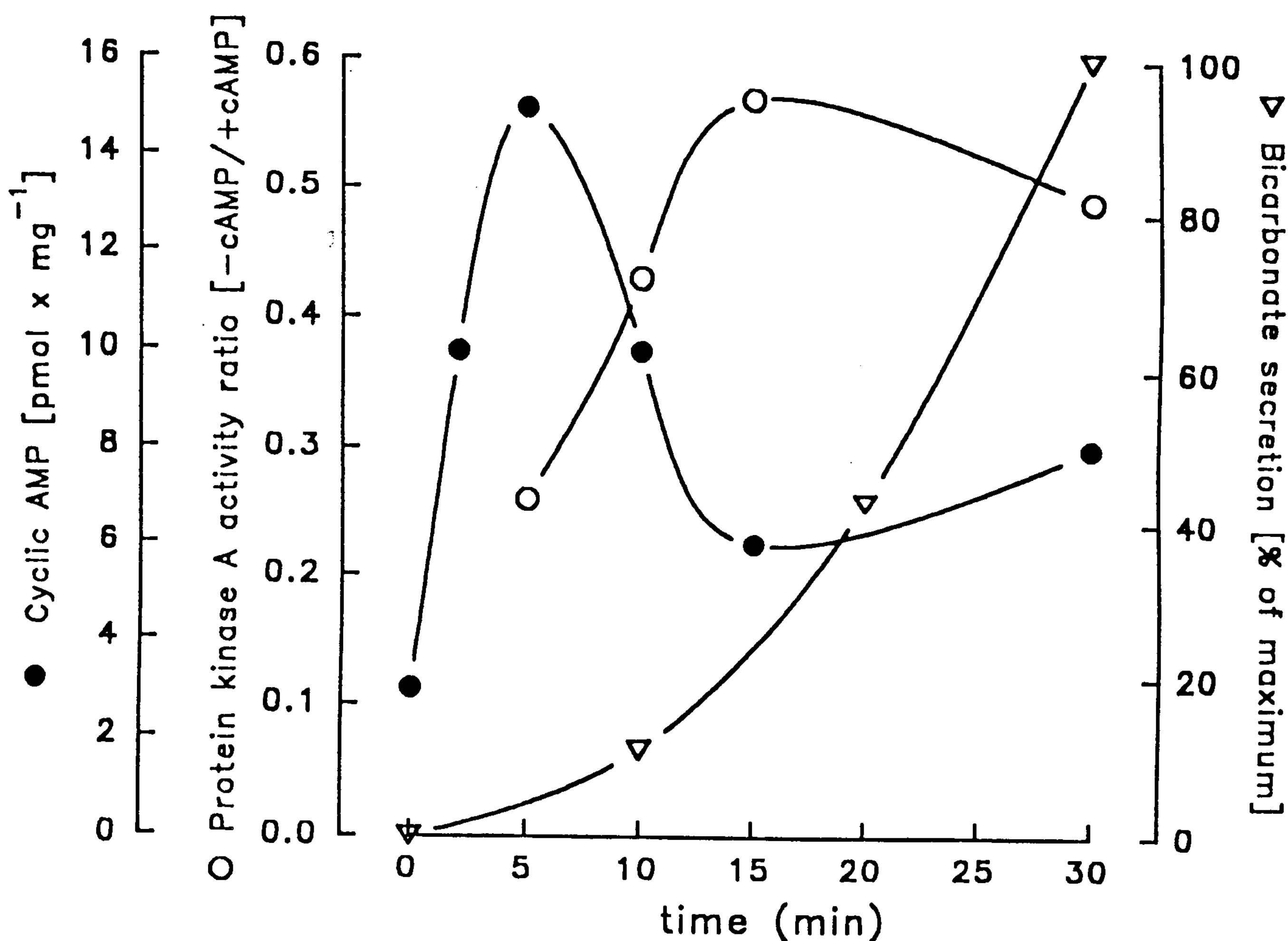


FIGURE 6. Time-course of effects after VIP treatment in the guinea-pig proximal duodenum. In <u>one experiment</u>, isolated duodenal enterocytes were incubated in the presence of VIP (10^{-7} mol/l); at the indicated time points, an aliquot of cells was removed and intracellular cAMP and protein kinase A activity were determined. In a <u>separate experiment</u>, VIP (10^{-10} mol.kg^{-1}.30 min^{-1}) was administered i.v. to anaesthetized guinea-pigs and bicarbonate secretion from the perfused duodenal segment was measured. Values shown are representative of at least three different cell preparations or animals, which all gave similar results. All values are plotted on the same x-axis for <u>comparison of time-related events</u>.



relationship was found after stimulation in vitro and in vivo with PGE_2 in similar dosages. (Reimer et al. in press).

Combinations of Agonists: Evidence that Multiple Intracellular Messengers modulate Bicarbonate Secretion:

The data obtained above suggested that both the adenylate cyclase/protein kinase A cascade (activated by dBcAMP, VIP, PGE_2, theophylline) and the inositol phospholipid/protein kinase C cascade (activated by $PGF_{2\alpha}$, TPA, dBcGMP) participated in bicarbonate secretion. Experiments were then designed where two agonists were infused together to see whether additive effects on bicarbonate secretion would occur. The results appear in detail in Tables 3 to 9, and in Fig. 7.

The interaction of glucagon and VIP illustrates the type of experiment. After basal bicarbonate secretion was stable (8.1 ± 0.9 $\mu mol.cm^{-1}.10\ min^{-1}$ (note: 10 min!), glucagon was infused in the dose 10^{-6} $mol.kg^{-1}$ for 30 min. This was associated with a significant increase in the bicarbonate secretion above the basal ($P < 0.001$). Then, while the glucagon infusion continued, VIP 10^{-11} $mol.kg^{-1}$ was infused simultaneously, and this produced a significant drop of bicarbonate secretion, which returned to basal values ($P < 0.005$ versus glucagon alone). When VIP was discontinued and glucagon was infused on its own again, the

bicarbonate secretion rate returned to its former stimulated value (Fig. 7, Table 3). Therefore, VIP served to inhibit bicarbonate secretion, and this was not associated with changes in the blood pressure of the animals (local duodenal blood flow was not measured).

Similarly, PGE_2 (Table 4) reduced the stimulatory effect of glucagon on bicarbonate secretion.

However, $PGF_{2\alpha}$ increased the glucagon-stimulated bicarbonate output when these agents were infused together (Table 5). The bicarbonate outputs were as follows: basal 10 ± 1 $\mu mol.cm^{-1}.30\ min^{-1}$, glucagon alone 14 ± 1 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.005$ versus basal), glucagon plus $PGF_{2\alpha}$ 18.2 ± 2 ($P < 0.005$ versus glucagon).

When VIP and PGE_2 were infused together, the bicarbonate output fell below the basal value ($P < 0.001$, Table 6). However, the combination of VIP with $PGF_{2\alpha}$ increased bicarbonate secretion above the stimulated value of VIP on its own ($P < 0.005$, Table 7).

The effect of infusing PGE_2 and $PGF_{2\alpha}$ together is shown in Table 8. PGE_2 stimulated the bicarbonate output above the basal value ($P < 0.005$). The bicarbonate secretion during infusion of both PGE_2 and $PGF_{2\alpha}$ was greater than during treatment with PGE_2 alone ($P < 0.025$).

FIGURE 7. Effect of VIP on glucagon-stimulated duodenal bicarbonate secretion. After basal bicarbonate secretion was measured for 30 min, glucagon (10^{-6} $mol.kg^{-1}$) was infused i.v. for 90 min. VIP (10^{-11} $mol.kg^{-1}$) was infused i.v. simultaneously during the time period indicated. Values are mean ± SEM from 5 animals. * P at least < 0.05 for differences from basal.

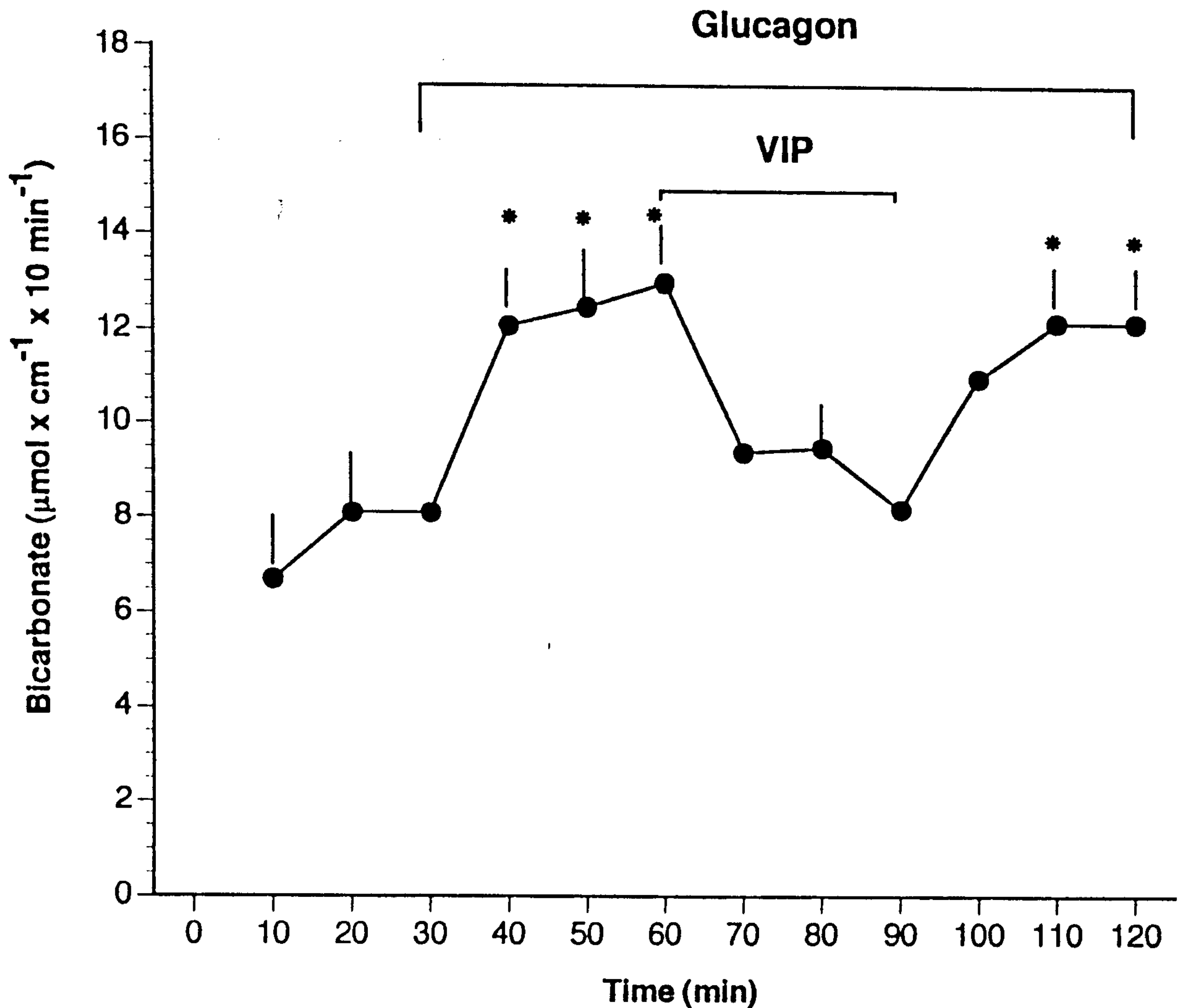


TABLE 3. Effect of combined i.v. infusion of glucagon and VIP on duodenal bicarbonate secretion

	HCO_3^- Secretion	
	$\mu mol.cm^{-1}.30\ min^{-1}$	% of basal
basal	26 ± 1	100
glucagon	41 ± 1*	156 ± 5*
glucagon + VIP	27 ± 2**	105 ± 5**
glucagon	41 ± 1*	159 ± 3*

Doses of the agonists: glucagon: 10^{-6} $mol.kg^{-1}.30\ min^{-1}$; VIP: 10^{-11} $mol.kg^{-1}.30\ min^{-1}$. Glucagon was infused for 3 periods of 30 min each, in sequence; VIP was infused simultaneously during the second period of glucagon-infusion. Values are mean ± SEM, n = 5.

* $P < 0.001$ greater than basal

** $P < 0.005$ versus glucagon alone

Note: While it is usual to depict the interactions between pharmacologic agents on isobolograms, the data were tabulated instead (Tables 3 to 9) since full dose relationships of these agents were not determined.

TABLE 4. Effect of combined i.v. infusion of glucagon and PGE_2 on duodenal bicarbonate secretion

	HCO_3^- Secretion	
	$\mu mol.cm^{-1}.30\ min^{-1}$	% of basal
basal	24 ± 2	100
glucagon	43 ± 3*	183 ± 17*
glucagon + PGE_2	29 ± 4**	126 ± 19**
glucagon	46 ± 3*	194 ± 10*

Doses of the agonists: glucagon: $10^{-6}\ mol.kg^{-1}.30\ min^{-1}$; PGE_2: $10^{-7}\ mol.kg^{-1}.30\ min^{-1}$. Glucagon was infused for 3 periods of 30 min each, in sequence; PGE_2 was infused simultaneously during the second period of glucagon-infusion. Values are mean ± SEM, n = 5.

* $P < 0.01$ greater than basal

** P NS versus basal, $P < 0.005$ less than glucagon alone

TABLE 5. Effect of combined i.v. infusion of glucagon and $PGF_{2\alpha}$ on duodenal bicarbonate secretion

	HCO_3^- Secretion	
	$\mu mol.cm^{-1}.30\ min^{-1}$	% of basal
basal	10 ± 1	100
glucagon	$14 \pm 1^{*}$	$142 \pm 7^{*}$
glucagon + $PGF_{2\alpha}$	$18 \pm 2^{**}$	$185 \pm 12^{**}$
glucagon	$13 \pm 1^{*}$	$135 \pm 7^{*}$

Doses of the agonists: glucagon: 10^{-6} $mol.kg^{-1}.30\ min^{-1}$; $PGF_{2\alpha}$: 10^{-6} $mol.kg^{-1}.30\ min^{-1}$. Glucagon was infused for 3 periods of 30 min each, in sequence; $PGF_{2\alpha}$ was infused simultaneously during the second period of glucagon-infusion. Values are mean ± SEM, n = 6.

* $p < 0.005$ greater than basal

** $p < 0.005$ greater than glucagon alone

TABLE 6. Effect of combined i.v. infusion of VIP and PGE_2 on duodenal bicarbonate secretion

	HCO_3^- Secretion	
	$\mu mol.cm^{-1}.30\ min^{-1}$	% of basal
basal	29 ± 2	100
VIP	41 ± 2*	140 ± 6*
VIP + PGE_2	12 ± 2**	39 ± 4**
VIP	36 ± 2*	124 ± 4*

Doses of the agonists were: VIP: 10^{-11} $mol.kg^{-1}.30\ min^{-1}$; PGE_2: 10^{-7} $mol.kg^{-1}.30\ min^{-1}$. VIP was infused for 3 periods of 30 min each, in sequence; PGE_2 was infused simultaneously during the second period of VIP-infusion. Values are mean ± SEM, n = 6.

* $P < 0.005$ greater than basal

** $P < 0.001$ versus basal, $P < 0.001$ versus VIP alone

TABLE 7. Effect of combined i.v. infusion of VIP and PGF_{2a} on duodenal bicarbonate secretion

	HCO_3^- Secretion	
	$\mu mol.cm^{-1}.30\ min^{-1}$	% of basal
basal	27 ± 1	100
VIP	39 ± 1*	142 ± 4*
VIP + $PGF_{2\alpha}$	47 ± 1**	171 ± 4**
VIP	40 ± 1*	147 ± 3*

Doses of the agonists: VIP: 10^{-11} $mol.kg^{-1}.30\ min^{-1}$; $PGF_{2\alpha}$: 10^{-6} $mol.kg^{-1}.30\ min^{-1}$. VIP was infused for 3 periods of 30 min each, in sequence; $PGF_{2\alpha}$ was infused simultaneously during the second period of VIP-infusion. Values are mean ± SEM, n = 7.

* $P < 0.001$ greater than basal

** $P < 0.005$ greater than VIP alone

TABLE 8. Effect of combined i.v. infusion of PGE_2 and PGF_{2a} on duodenal bicarbonate secretion

	HCO_3^- Secretion	
	$\mu mol.cm^{-1}.30\ min^{-1}$	% of basal
basal	27 ± 1	100
PGE_2	38 ± 2*	146 ± 8*
$PGE_2 + PGF_{2\alpha}$	48 ± 3**	184 ± 11**
PGE_2	38 ± 2*	145 ± 6*

Doses of the agonists: PGE_2: $10^{-7}\ mol.kg^{-1}.30\ min^{-1}$; $PGF_{2\alpha}$: $10^{-6}\ mol.kg^{-1}.30\ min^{-1}$. PGE_2 was infused for 3 periods of 30 min each, in sequence; $PGF_{2\alpha}$ was infused simultaneously during the second period of PGE_2-infusion. Values are mean ± SEM, n = 6.

* $P < 0.005$ greater than basal

** $P < 0.025$ greater than PGE_2 alone

TABLE 9. Effect of dBcAMP, PGE_2 and VIP, given alone and in combination, on duodenal bicarbonate secretion

	Duodenal HCO_3^- secretion (% of basal)
Basal	100
PGE_2	277 ± 21 *
VIP	229 ± 15 *
dBcAMP	366 ± 16 *
dBcAMP + PGE_2	252 ± 1 **
dBcAMP + VIP	304 ± 18 ***

dBcAMP (5 x 10^{-8} mol.kg^{-1}), PGE_2 (5 x 10^{-7} mol.kg^{-1}) and VIP (5 x 10^{-9} mol.kg^{-1}) were injected i.v. in separate veins over 5 min, alone or in the combinations indicated. Duodenal bicarbonate secretion was collected over a 30 min period. Values are mean ± SEM, n = 4.

* significantly higher than basal ($P < 0.005$)

** significantly lower than dBcAMP alone ($P < 0.005$)

*** P = NS versus dBcAMP alone

PGE_2 and VIP were shown to reduce the stimulatory effect of dBcAMP on bicarbonate secretion ($P < 0.005$), although the
level of secretion was still greater than the basal output (Table 9).

These data therefore indicate that the combination, in submaximal doses, of two agonists, which both utilize the adenylate cyclase and protein kinase A cascade, leads to reduced bicarbonate output than when one agonist of the pair is administered alone (examples: glucagon and VIP, glucagon and PGE_2, VIP and PGE_2, dBcAMP and PGE_2, dBcAMP and VIP). Certainly, there was no additive effect, as we had anticipated.

However, there was a summative effect when an agonist acting in the adenylate cyclase/protein kinase A cascade was combined with an agonist acting the inositol phospholipid and protein kinase C cascade (example: VIP and $PGE_{2\alpha}$, PGE_2 and $PGF_{2\alpha}$, glucagon and $PGF_{2\alpha}$). To this must be added the observation that carbachol also stimulated bicarbonate secretion.

CHOLINERGIC PATHWAYS OF BICARBONATE SECRETION

Effect of Cholinergic Stimulation of Basal Bicarbonate Secretion:

Basal bicarbonate output was steady at 13.7 ± 0.5 $\mu mol.cm^{-1}.30\ min^{-1}$.

Following the i.v. injection of 0.2 $\mu g.kg^{-1}.5\ min^{-1}$ (10^{-9} $mol.kg^{-1}.5\ min^{-1}$) carbachol, the bicarbonate output rose abruptly to 28.8 ± 1.6 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.001$ versus basal) and then returned to basal by 30 min (Fig. 8).

Carbachol 2 $\mu g.kg^{-1}.5\ min^{-1}$ (10^{-8} $mol.kg^{-1}.5\ min^{-1}$) produced a significantly greater increase in bicarbonate secretion to 35.6 ± 1.7 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.001$ versus basal, and versus the lower carbachol dose).

Although the order of the two carbachol doses was randomized, the stimulatory effect of each dose was reproducible (unpaired t test showed no statistical difference between the bicarbonate outputs in 3 animals receiving the lower and then the higher carbachol dose, and 2 animals receiving the carbachol doses in reverse order).

Effect of Muscarinic Blockade of Basal Bicarbonate Secretion:

The effect of muscarinic inhibition by atropine and pirenzepine on basal duodenal bicarbonate secretion was studied first.

Atropine 0.1 $mg.kg^{-1}.5\ min^{-1}$ (equal to 1.5×10^{-7} $mol.kg^{-1}.5\ min^{-1}$) i.v. reduced the bicarbonate secretion from basal 10.2 ± 1.2 $\mu mol.cm^{-1}.30\ min^{-1}$ to 4.1 ± 2.7 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.05$).

Pirenzepine 1 $mg.kg^{-1}.5\ min^{-1}$ (equalling 2×10^{-6} $mol.kg^{-1}.5\ min^{-1}$) i.v. similarly diminished the duodenal bicarbonate secretion from basal 12.3 ± 1.3 $\mu mol.cm^{-1}.30\ min^{-1}$ to -3.3 ± 1.3 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.01$). The "negative" value -3.3 ± 1.3 $\mu mol.cm^{-1}.30\ min^{-1}$ indicates absorption of bicarbonate from the hyperconcentrated perfusate in the duodenal lumen.

Effect of Muscarinic Blockade of Stimulated Bicarbonate Secretion:

The effect of m-cholinoceptor blockade on carbachol-stimulated duodenal bicarbonate secretion was studied next.

Three doses of carbachol (2 $\mu g.kg^{-1}.5\ min^{-1}$, equal to 10^{-8} $mol.kg^{-1}.5\ min^{-1}$) were infused i.v. consecutively at 40 min intervals. Atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$) or pirenzepine

FIGURE 8. Effect of carbachol i.v. infusion on duodenal bicarbonate secretion in the anaesthetized guinea-pig. Carbachol was infused in two doses, 0.2 $\mu g.kg^{-1}.5\ min^{-1}$ and 2.0 $\mu g.kg^{-1}.5\ min^{-1}$ (corresponding to $10^{-9}\ mol.kg^{-1}.5\ min^{-1}$ and $10^{-8}\ mol.kg^{-1}.5\ min^{-1}$), at 40 min intervals. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 5 animals. * P at least < 0.05 versus basal.

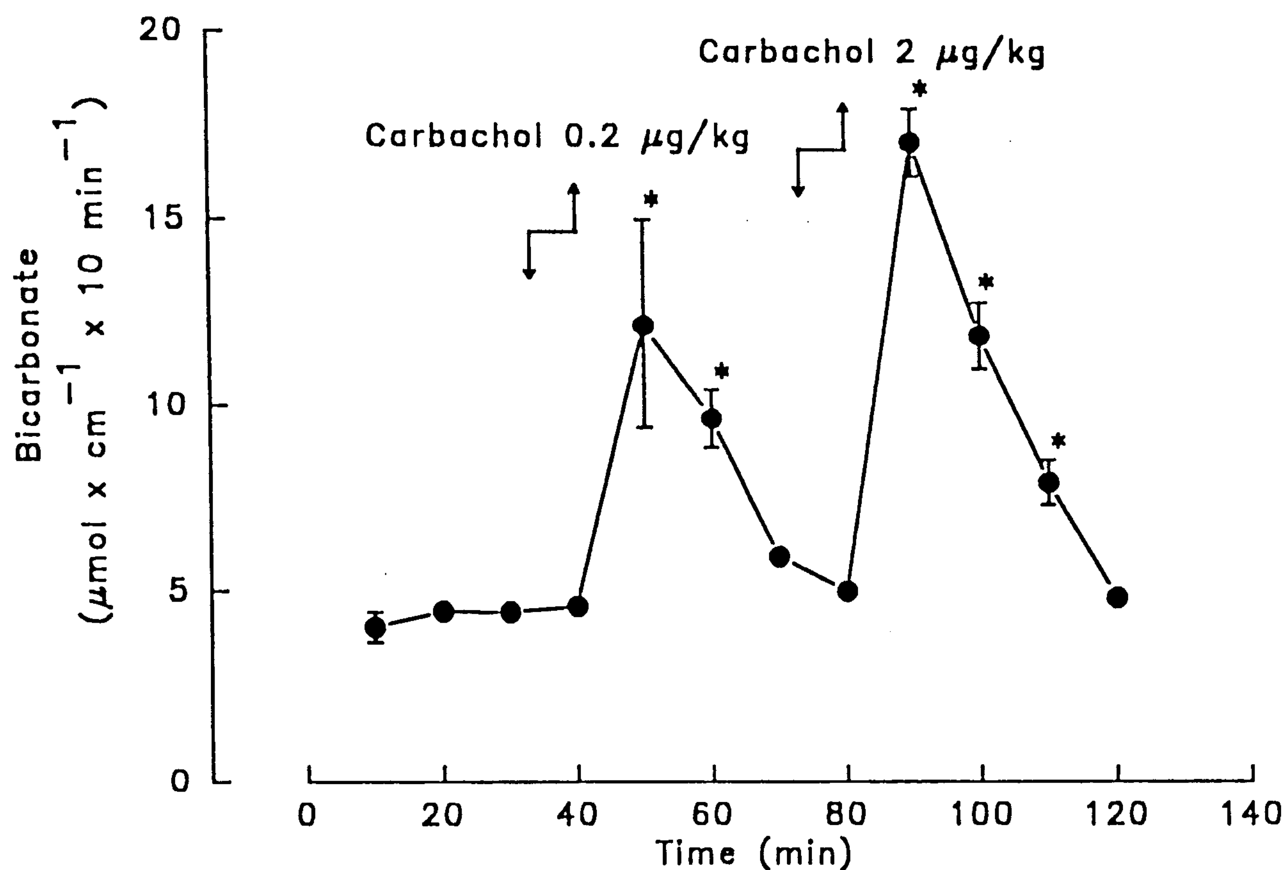


TABLE 10. Effect of atropine, pirenzepine and hexamethonium on carbachol-stimulated bicarbonate output

	Basal	Carbachol	Carbachol + Antagonist
	Bicarbonate (μmol.cm^{-1}.30 min^{-1})		
Atropine (0.1 mg)	11 ± 0.1	35 ± 1.5 [a]	7 ± 1.0 [b,c]
Pirenzepine (1 mg)	11 ± 0.5	33 ± 3.2 [b]	9 ± 0.7 [d,e]
Hexamethonium (0.3 mg)	8 ± 0.3	23 ± 1.0 [a]	6 ± 0.3 [d,e]

All drugs (doses/kg) were infused i.v. over 5 min. Bicarbonate secretion stimulated by carbachol given alone at the conclusion of each experiment was not significantly different from that produced by the first infusion of carbachol alone (data not shown). Values are mean ± SEM from at least 4 animals.

[a] $P < 0.001$ vs basal

[b] $P < 0.005$ vs basal

[c] $P < 0.001$ vs carbachol

[d] $P < 0.025$ vs basal

[e] $P < 0.005$ vs carbachol

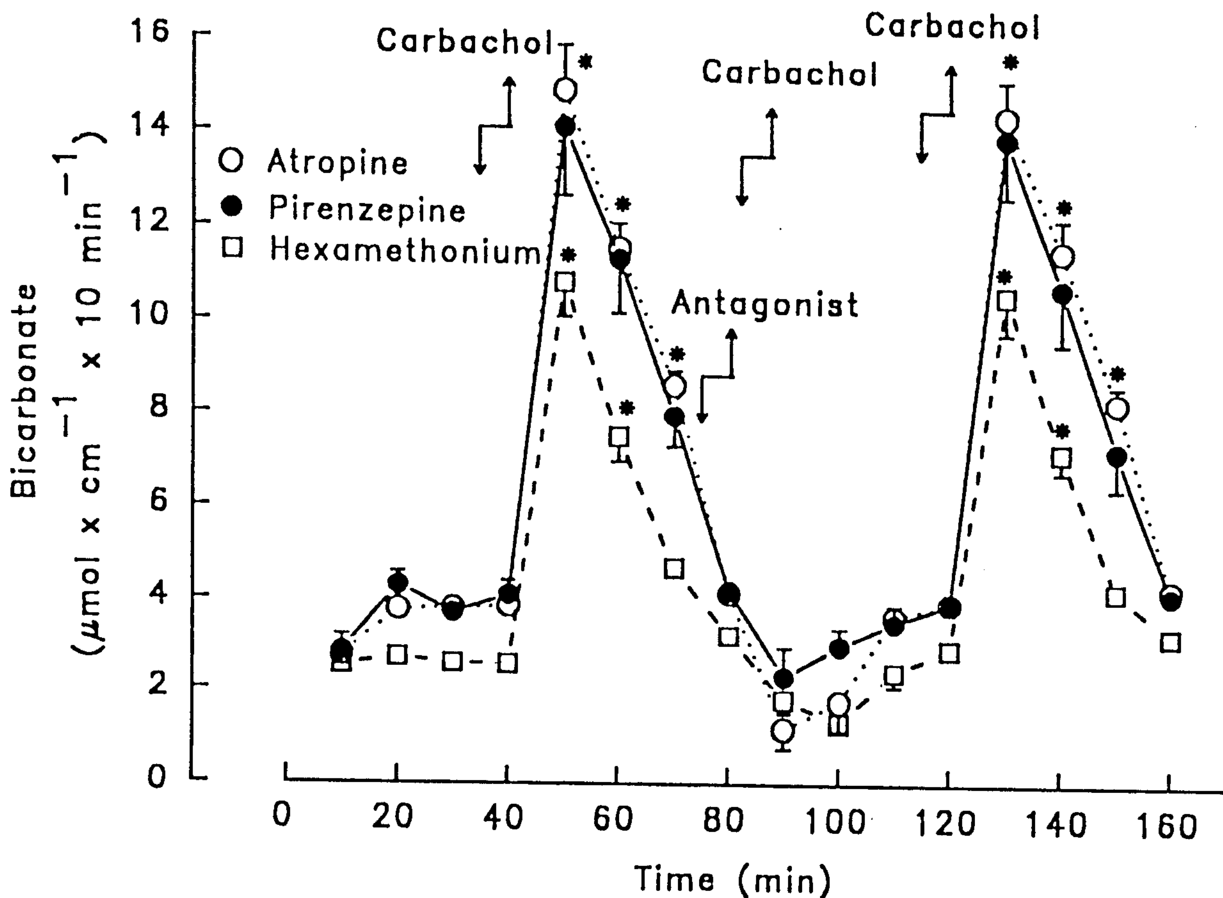
(1 $mg.kg^{-1}.5\ min^{-1}$) was injected 5 min prior to the second carbachol dose. Both drugs abolished the stimulatory effect of carbachol and reduced the bicarbonate output to values below basal (Fig. 9, Table 10).

In the doses used, the effects of atropine and pirenzepine on carbachol-stimulated duodenal bicarbonate secretion were similar. Furthermore, the effect of each drug was short-lived.

Effect of Nicotinic Blockade on Bicarbonate Secretion

Hexamethonium in the dose 0.3 $mg.kg^{-1}.5\ min^{-1}$ (10^{-6} $mol.kg^{-1}.5\ min^{-1}$) given by i.v. infusion significantly inhibited the stimulatory effect of 2 $\mu g.kg^{-1}.5\ min^{-1}$ carbachol on duodenal bicarbonate secretion ($P < 0.001$) (Fig. 9 and Table 10).

FIGURE 9. Effects of atropine (0.1 mg.kg^{-1}.5 min^{-1}, corresponding to 1.5 x 10^{-7} mol.kg^{-1}.5 min^{-1}), pirenzepine (1.0 mg.kg^{-1}.5 min^{-1}, corresponding to 2 x 10^{-6} mol.kg^{-1}.5 min^{-1}) or hexamethonium (0.3 mg.kg^{-1}.5 min^{-1}, corresponding to 10^{-6} mol.kg^{-1}.5 min^{-1}) on carbachol-stimulated duodenal bicarbonate secretion in anaesthetized guinea-pigs. Carbachol (2 μg.kg^{-1}.5 min^{-1}, corresponding to 10^{-8} mol.kg^{-1}.5 min^{-1}) was infused i.v. at 40 min intervals on three occasions. The antagonists were infused i.v. 5 min before the second dose of carbachol. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 6 animals (atropine and pirenzepine) and n = 4 animals (hexamethonium). * P at least < 0.05 compared with basal.



In these experiments there was a sharp drop of blood pressure (from mean 110 to mean 50 mm Hg) upon injection of hexamethonium, followed by slow recovery over the succeeding 60 min. Therefore, the reduction of bicarbonate output to below basal occurred during the period of hypotension (systolic blood pressure of 50 mm Hg).

Rapid intravenous infusion of physiological solution failed to prevent or correct this hypotension.

Role of VIP in the Cholinergic Stimulation of Bicarbonate Secretion:

The effect of the VIP-antagonist [D-p-Cl-Phe^6,Leu^{17}]-VIP on carbachol-stimulated bicarbonate secretion was studied.

Duodenal bicarbonate secretion was 9.4 $\pm$ 0.8 $\mu mol.cm^{-1}.30\ min^{-1}$ in the basal state and 8.0 $\pm$ 0.9 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.025$) during infusion of the VIP-antagonist [D-p-Cl-Phe^6,Leu^{17}]-VIP (10^{-6} $mol.kg^{-1}$, or 3.3 $mg.kg^{-1}$). Simultaneous infusion of VIP-antagonist and carbachol (2 $\mu g.kg^{-1}$) reduced the bicarbonate secretion to 5.0 $\pm$ 0.4 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.005$ versus basal; the reason for this is not known). Discontinuation of the infusion of VIP-antagonist resulted in a 3-fold increase in bicarbonate secretion in response to carbachol alone, 14.3 $\pm$ 0.6 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.025$).

Effect of Muscarinic Blockade on VIP-Stimulated Bicarbonate Secretion:

In a further experiment, the basal bicarbonate was measured as 8.6 ± 0.1 $\mu mol.cm^{-1}.30\ min^{-1}$. VIP (5 $\mu g.kg^{-1}$) was infused for 3 periods of 30 minutes, and pirenzepine (1 $mg.kg^{-1}.5\ min^{-1}$) was injected prior to the second VIP treatment. Bicarbonate secretion was: 16.2 ± 0.9, 16.6 ± 1.0 and 15.9 ± 0.6 $\mu mol.cm^{-1}.30\ min^{-1}$ respectively (P = NS, Table 11).

In another experiment, atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$) was injected in place of pirenzepine during VIP-stimulated bicarbonate secretion. Bicarbonate outputs were as follows: basal 7.6 ± 0.4 $\mu mol.cm^{-1}.30\ min^{-1}$, atropine and VIP infusion 12.0 ± 0.8 $\mu mol.cm^{-1}.30\ min^{-1}$ (P = NS versus basal, n = 3), VIP (alone) infusion 18.5 ± 0.9 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.025$ versus basal secretion, Table 11).

Subtracting the basal bicarbonate secretion from atropine plus VIP-induced secretion yielded a value of 4.4 ± 1.2 $\mu mol.cm^{-1}.30\ min^{-1}$. Subtracting the basal bicarbonate from VIP-induced secretion yielded 10.9 ± 1.2 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.01$ versus the atropine plus VIP). This implies that atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$) reduced VIP-stimulated bicarbonate secretion by about 60%.

TABLE 11. Effect of muscarinic blockade by pirenzepine and atropine on VIP-stimulated duodenal bicarbonate secretion

	Pirenzepine	Atropine
	Bicarbonate secretion ($\mu mol.cm^{-1}.30\ min^{-1}$)	
Basal	8.6 ± 0.1	7.6 ± 0.4
VIP	16.2 ± 0.9 [a]	ND
VIP + antagonist	16.6 ± 1.0 [a]	12.0 ± 0.8 [b]
VIP	15.9 ± 0.6 [a]	18.5 ± 0.9 [c]

Doses were: pirenzepine 1 $mg.kg^{-1}$, atropine 0.1 $mg.kg^{-1}$, VIP 5 $\mu g.kg^{-1}$.

[a] $P < 0.05$ versus basal

[b] P = NS versus basal

[c] $P < 0.025$ versus basal

Effect of Muscarinic Blockade on PGE_2-Stimulated Bicarbonate Secretion:

Pirenzepine (1 $mg.kg^{-1}.5\ min^{-1}$) had no effect on PGE_2 (10^{-7} mol/kg)-stimulated bicarbonate secretion. In this study, basal bicarbonate secretion was 8.6 ± 0.4 $\mu mol.cm^{-1}.30\ min^{-1}$. PGE_2 increased the bicarbonate secretion sharply to 31.1 ± 2.1 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.001$). During PGE_2 plus pirenzepine infusion, the bicarbonate production was

31.2 ± 0.6 $\mu mol.cm^{-1}.30\ min^{-1}$. In the final period when PGE_2 was infused alone, the bicarbonate production was 28.8 ± 0.4 $\mu mol.cm^{-1}.30\ min^{-1}$ (P = NS for these values).

Similarly, atropine (0.1 $mg.kg^{-1}.5\ min^{-1}$) had no effect on PGE_2-stimulated bicarbonate secretion (data not shown, but similar to pirenzepine data).

The effect of hexamethonium on PGE_2-stimulated bicarbonate secretion was not tested, since the hypotensive effects of this drug make the results questionable.

ROLE OF INTRACELLULAR Ca^{2+} IN THE CHOLINERGIC STIMULATION OF BICARBONATE SECRETION

Effects of Verapamil and Ca^{2+} Ionophore A23187 on Basal Bicarbonate Secretion:

The calcium-channel blocking agent verapamil and the Ca^{2+} ionophore A23187 were infused i.v. to study their effect on basal duodenal bicarbonate secretion.

FIGURE 10. Effect of A23187 on duodenal bicarbonate secretion in anaesthetized guinea-pigs. The ionophore (5, 50, 500 $\mu g.kg^{-1}.5\ min^{-1}$, corresponding to 10^{-8}, 10^{-7} and 10^{-6} $mol.kg^{-1}.5\ min^{-1}$) was infused i.v. over 5 min every 40 min. DMSO (the vehicle of A23187) had no effect. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 6 animals. * $P < 0.05$ compared with basal.

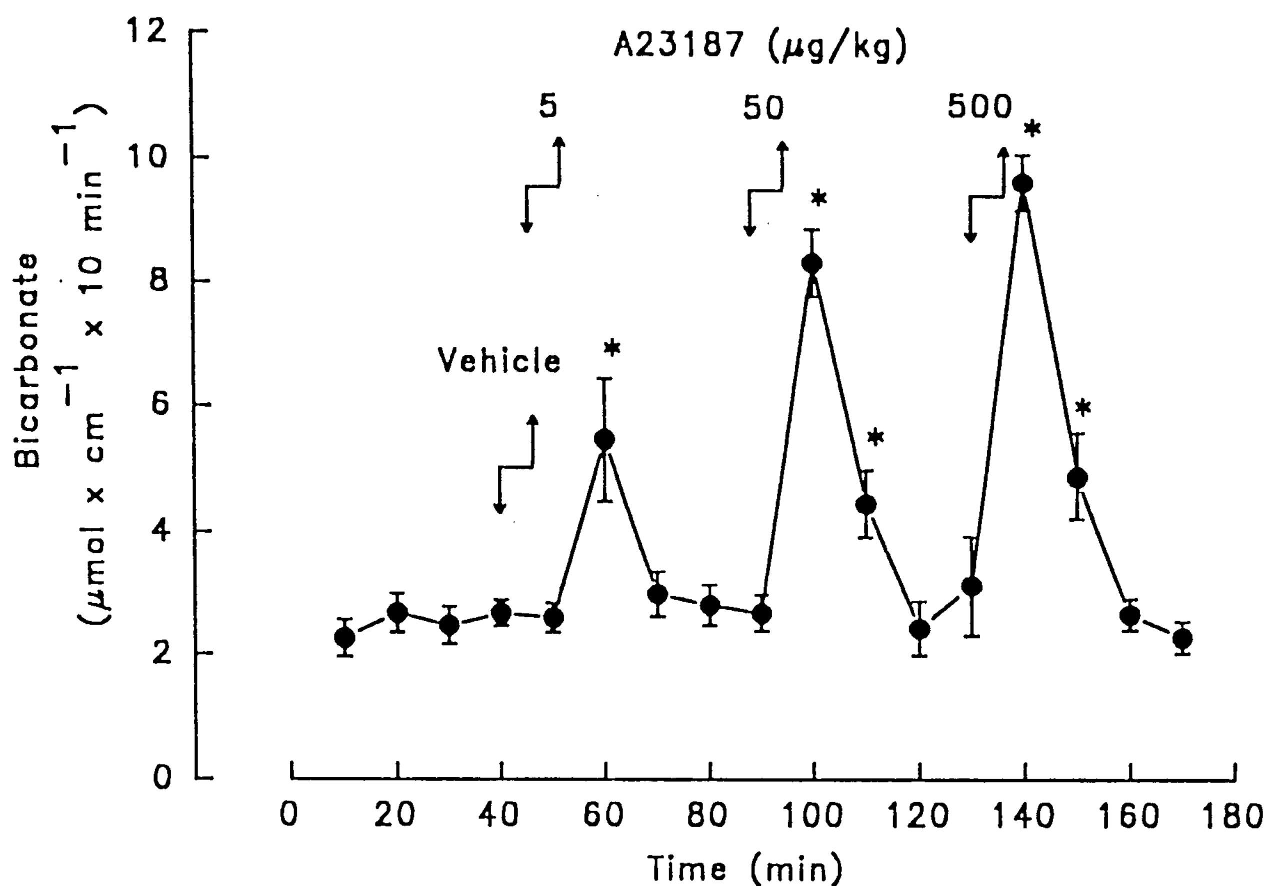
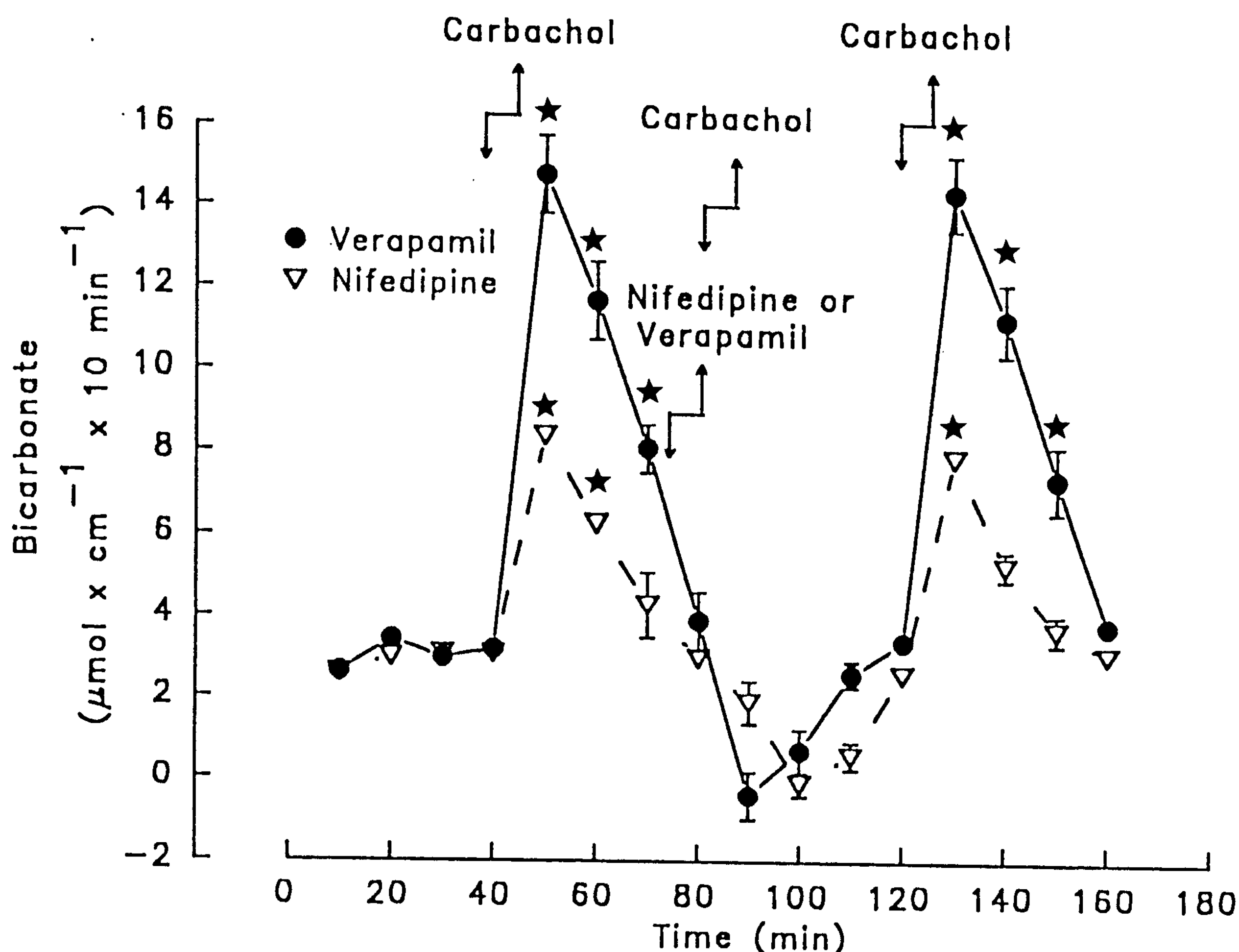


FIGURE 11. Effect of verapamil (0.2 mg.kg^{-1}.5 min^{-1}, corresponding to 4 x 10^{-7} mol.kg^{-1}.5 min^{-1}) or nifedipine (1.7 mg.kg^{-1}.5 min^{-1}, corresponding to 5 x 10^{-6} mol.kg^{-1}.5 min^{-1}) on carbachol-stimulated duodenal bicarbonate secretion in anaesthetized guinea-pigs. Carbachol (2 μg.kg^{-1}.5 min^{-1}, corresponding to 10^{-8} mol.kg^{-1}.5 min^{-1}) was infused i.v. at 40 min intervals on three occasions. Verapamil or nifedipine was infused i.v. 5 min before the second injection of carbachol. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 6 animals. * P at least < 0.05 compared with basal.



Verapamil (0.2 mg.kg^{-1}.5 min^{-1}) decreased the duodenal bicarbonate secretion from a basal value of 10.8 $\pm$ 0.4 μmol.cm^{-1}.30 min^{-1} to 3.1 $\pm$ 1.5 μmol.cm^{-1}.30 min^{-1} ($P < 0.01$).

Calcium ionophore A23187 in the dose range 5 to 500 μg.kg^{-1}.5 min^{-1} increased bicarbonate secretion significantly (Fig. 10). The 30 min bicarbonate outputs after A23187 in the doses 5, 50 and 500 μg.kg^{-1}.5 min^{-1} were: 11.2 $\pm$ 1.4 μmol.
cm^{-1}.30 min^{-1}, 15.2 $\pm$ 1.1 μmol.cm^{-1}.30 min^{-1} and 17.2 $\pm$ 0.5 μmol.cm^{-1}.30 min^{-1} respectively, compared with basal 7.4 $\pm$ 0.6 μmol.cm^{-1}.30 min^{-1} ($P < 0.025$ for all changes). The vehicle (DMSO) of A23187 did not affect duodenal bicarbonate secretion (7.8 $\pm$ 0.7 μmol.cm^{-1}.30 min^{-1}).

Effects of Verapamil, Nifedipine and the Calcium Ionophore A23187 on Carbachol-Stimulated Bicarbonate Secretion:

The effect of modulating intracellular Ca^{2+} on carbachol-stimulated duodenal bicarbonate secretion was studied by infusing three doses of carbachol (2 μg/kg over 5 min) at 40 min intervals; verapamil (0.2 mg.kg^{-1}.5 min^{-1}), nifedipine (1.7 mg.kg^{-1}.5 min^{-1}) or A23187 (0.5 mg.kg^{-1}.5 min^{-1}) was injected i.v. just prior to the second carbachol dose. Verapamil and nifedipine both abolished completely the stimulatory effect of carbachol, and the duodenal

bicarbonate secretion was reduced to below basal with each drug (Fig. 11, Table 12).

By comparison, A23187 increased the bicarbonate secretion above that obtained with carbachol alone (Table 12). The basal bicarbonate secretion was 14.0 ± 0.5 μmol. cm^{-1}.30 min^{-1}, carbachol alone produced a bicarbonate output of 41.4 ± 1.0 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.05$ versus basal), carbachol plus A23187 produced a bicarbonate output of 46.6 ± 1.4 $\mu mol.cm^{-1}$ .30 min^{-1} ($P < 0.05$ versus carbachol alone), and carbachol alone at the end of the experiment produced a bicarbonate output of 40.3 ± 1.5 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.05$ versus basal).

The modest degree of the increase in carbachol-stimulated bicarbonate secretion induced by adding A23187 is due to the near maximum rate of bicarbonate secretion already achieved by carbachol alone.

Effect of VIP-antagonist on A23187-stimulated bicarbonate secretion:

The effect of [D-p-Cl-Phe^6,Leu^{17}]-VIP (3.3 $mg.kg^{-1}.30\ min^{-1}$) on A23187 (500 $\mu g.kg^{-1}.30\ min^{-1}$)-stimulated bicarbonate secretion was studied.

TABLE 12. Effect of verapamil, nifedipine and A23187 on carbachol-stimulated bicarbonate output

	Basal	Carbachol	Carbachol + agonist or antagonist
	Bicarbonate (μmol.cm^{-1}.30 min^{-1})		
Verapamil (0.2 mg)	12 ± 1.3	37 ± 3.3 [a]	5 ± 1.5 [b,c]
Nifedipine (1.7 mg)	12 ± 0.3	22 ± 0.8 [a]	5 ± 0.6 [c]
A23187 (0.5 mg)	10 ± 0.7	27 ± 4.8 [b]	31 ± 5.2 [d,e]

All drugs (doses/kg) were infused i.v. over 5 min. Bicarbonate secretion stimulated by carbachol infused given alone at the conclusion of each experiment (see methods) was not significantly different from that produced by the first infusion of carbachol alone (data not shown). Values are mean ± SEM from at least 4 animals.

[a] $p < 0.001$ vs basal
[b] $p < 0.01$ vs basal
[c] $p < 0.001$ vs carbachol
[d] $p < 0.005$ vs basal
[e] $p < 0.025$ vs carbachol

The bicarbonate output was 11.2 ± 0.6 $\mu mol.cm^{-1}.30\ min^{-1}$ under basal conditions, 10.8 ± 0.8 $\mu mol.cm^{-1}.30\ min^{-1}$ during treatment with [D-p-Cl-Phe^{6},Leu^{17}]-VIP, and 10.7 ± 0.6 $\mu mol.cm^{-1}.30\ min^{-1}$ during simultaneous treatment with [D-p-Cl-Phe^{6}, Leu^{17}]-VIP plus A23187 (P = NS for these changes). The bicarbonate output rose to 20.8 ± 0.7 $\mu mol.cm^{-1}.30\ min^{-1}$ during infusion of A23187 alone at the end of this experiment ($P < 0.001$).

M-Cholinoceptors and Intracellular Ca^{2+} in the Duodenal Enterocytes:

There was a virtual absence of ^{3}H-N-methylscopolamine binding and no rise of the intracellular Ca^{2+} concentration in response to carbachol stimulation of isolated duodenal enterocytes, as reported separately by Reimer et al. (1993a) who performed these measurements on our behalf.

This indicates that the duodenal enterocytes contain very few m-cholinoceptors and that carbachol stimulation of duodenal bicarbonate secretion requires an intermediary messenger. We have shown that this is the neuropeptide, VIP, which acted via cAMP and protein kinase A.

It should be noted, that protein kinase C also participates in bicarbonate secretion, as shown by the brisk response to stimulation of the duodenum in vivo with TPA.

EFFECT OF SOMATOSTATIN ON BICARBONATE SECRETION

Effect of Somatostatin on Basal Bicarbonate Secretion:

Basal bicarbonate output was 10.5 ± 0.5 $\mu mol.cm^{-1}.30\ min^{-1}$.

During the first intravenous infusion of somatostatin ($10^{-11}\ mol.kg^{-1}.30\ min^{-1}$), the bicarbonate output decreased progressively to -3.4 ± 6.1 $\mu mol.cm^{-1}.30\ min^{-1}$, the negative value indicating absorption of bicarbonate from the lumen ($P < 0.05$ vs. basal, Fig. 12). The subsequent infusions of larger doses of somatostatin (10^{-9}, 10^{-7} mole/kg) produced significantly greater negative values ($P < 0.05$ vs. preceding concentrations).

PGE_2, infused at the end of the experiment, stimulated the bicarbonate secretion to 17.2 ± 1.3 $mol.kg^{-1}.30\ min^{-1}$ ($P < 0.01$ vs. basal).

Effect of Somatostatin on Carbachol-Stimulated Bicarbonate Secretion:

Carbachol ($10^{-8}\ mol.kg^{-1}.30\ min^{-1}$) increased the bicarbonate secretion from basal 9.0 ± 0.5 to 23.3 ± 2.7 $\mu mol.cm^{-1}.30\ min^{-1}$ ($P < 0.025$, Fig. 13).

During infusion of somatostatin (10^{-7} mol/kg), the bicarbonate output was 3.0 ± 2.2 μmol.cm^{-1}.30 min^{-1} ($P < 0.001$ versus carbachol alone). When carbachol was again infused on its own, the secretion of bicarbonate rose to its previous stimulated level (21.4 ± 2.8 μmol.cm^{-1}.30 min^{-1}).

Effect of Somatostatin on VIP-Stimulated Bicarbonate Secretion:

VIP (10^{-8} mol.kg^{-1}.30 min^{-1}) increased the bicarbonate secretion from basal 9.5 ± 0.5 to 19.9 ± 1.4 μmol.cm^{-1}.30 min^{-1} ($P < 0.005$, Fig. 13). During somatostatin infusion (10^{-7} mol/kg), the bicarbonate output was 6.4 ± 3.9 μmol.cm^{-1}.30 min^{-1} ($P < 0.05$ versus VIP alone).

In the subsequent period when VIP alone was infused, the bicarbonate secretion was 20.6 ± 2.7 μmol.cm^{-1}.30 min^{-1}.

FIGURE 12. Effect of somatostatin-14 (10^{-11} mol.kg^{-1}, 10^{-9} mol.kg^{-1} and 10^{-7} mol.kg^{-1}) on basal duodenal bicarbonate secretion. Each dose was infused i.v. for 30 min. At the end of the study, PGE_2 (10^{-6} mol.kg^{-1}) was infused i.v. to test the secretory capacity of the duodenum. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 8 animals. * $P < 0.05$ compared with basal.

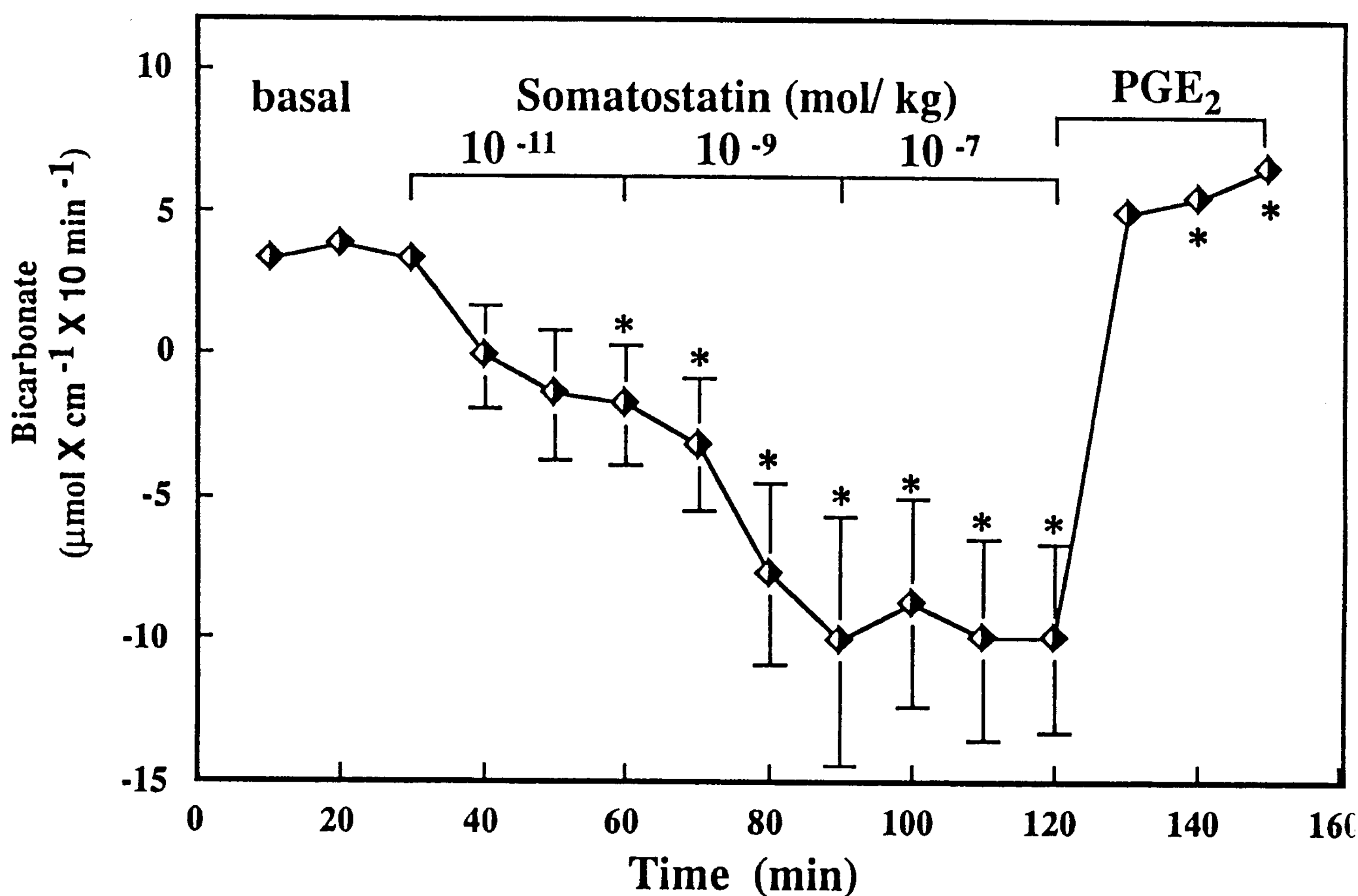
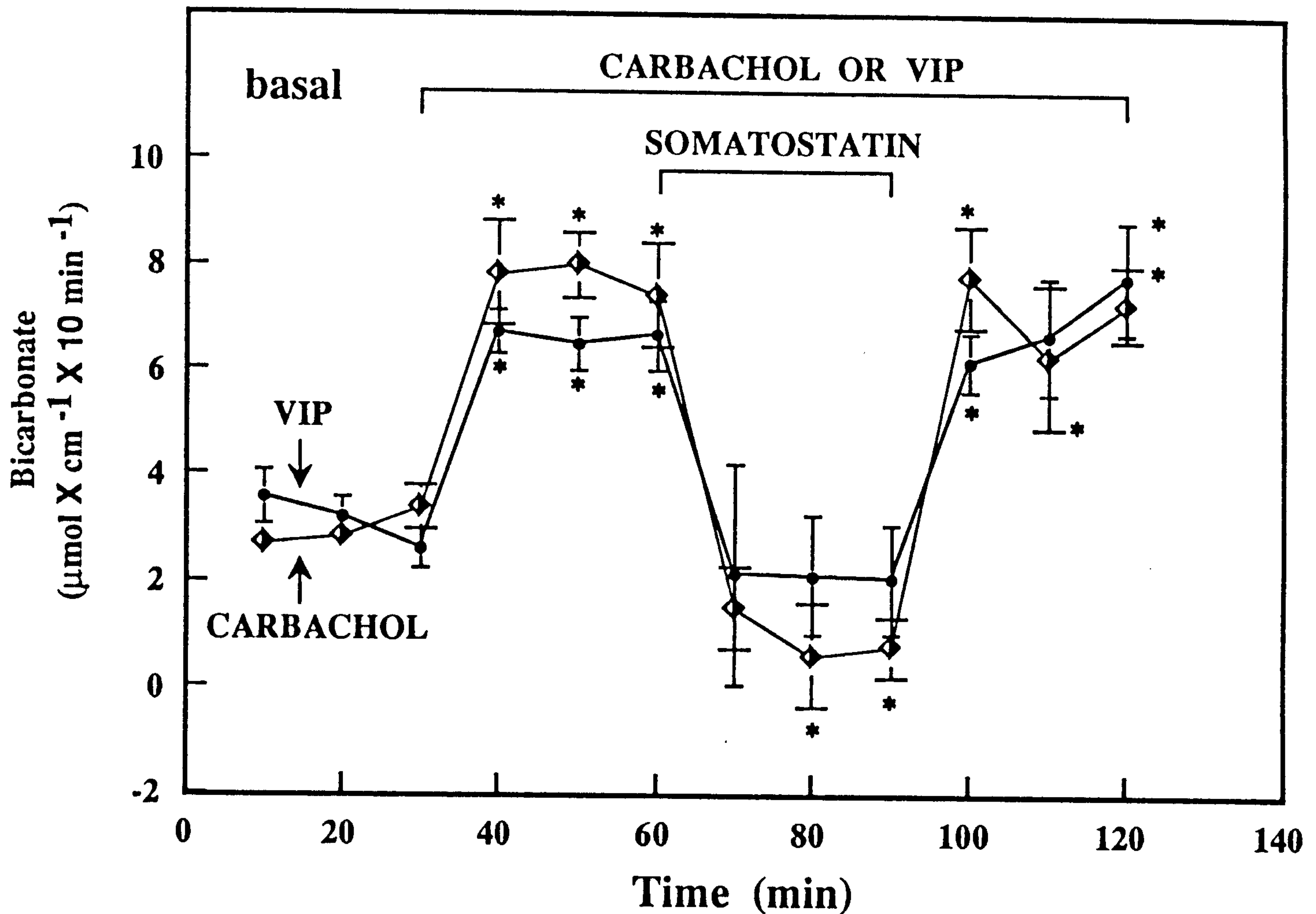


FIGURE 13. Effect of somatostatin-14 (10^{-7} mol.kg^{-1}) on duodenal bicarbonate secretion stimulated by carbachol (10^{-8} mol.kg^{-1}) and VIP (10^{-8} mol.kg^{-1}). Carbachol or VIP was infused i.v. in three consecutive periods of 30 min, and somatostatin was infused during the second 30 min period. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 4 animals. * $p < 0.05$ compared with basal.



Effect of Somatostatin on PGE_2-Stimulated Bicarbonate Secretion:

PGE_2 (10^{-8} mol.kg^{-1}.30 min^{-1}) increased the bicarbonate secretion from basal of 8.9 ± 0.2 μmol.cm^{-1}.30 min^{-1} to 21.5 ± 1.1 μmol.cm^{-1}.30 min^{-1} ($P < 0.001$, Fig. 14).

The bicarbonate output during the period of combined infusion with somatostatin (10^{-7} mol/kg) was 23.0 ± 2.0 μmol.cm^{-1}.30 min^{-1}.

Subsequently, when PGE_2 was again infused on its own, the bicarbonate output was 22.7 ± 1.2 μmol.cm^{-1}.30 min^{-1} (P = NS).

Somatostatin had no effect on PGE_2-stimulated duodenal bicarbonate secretion.

Effect of Somatostatin on Duodenal Enterocytic Adenylate Cyclase Activity:

Adenylate cyclase activity in homogenates of duodenal enterocytes (Table 13) was 9.4 ± 1.0 pmol cAMP.mg^{-1}.min^{-1} in the basal state, and 9.1 ± 1.7 pmol cAMP.mg^{-1}.min^{-1} in the presence of somatostatin (10^{-6} mol/l).

VIP (10^{-8} mol/l) and PGE_2 (10^{-7} mol/l) stimulated basal adenylate cyclase activity to 21.8 ± 3.6 and 30.9 ± 5.3 pmol.mg^{-1}.min^{-1}, respectively. There was no detectable

effect of somatostatin on PGE_2-stimulated and VIP-stimulated adenylate cyclase activity (Table 13).

Carbachol in a high dose (10^{-3} mol/l) had no affect on basal adenylate cyclase activity, therefore the combination of carbachol with somatostatin was not tested.

FIGURE 14. Effect of somatostatin-14 (10^{-7} mol.kg^{-1}) on duodenal bicarbonate secretion stimulated by PGE_2 (10^{-8} mol.kg^{-1}). PGE_2 was infused i.v. in three consecutive periods of 30 min, and somatostatin was infused during the second 30 min period. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Values are mean ± SEM for n = 4 animals. Somatostatin did not affect PGE_2-stimulated HCO_3^- secretion.

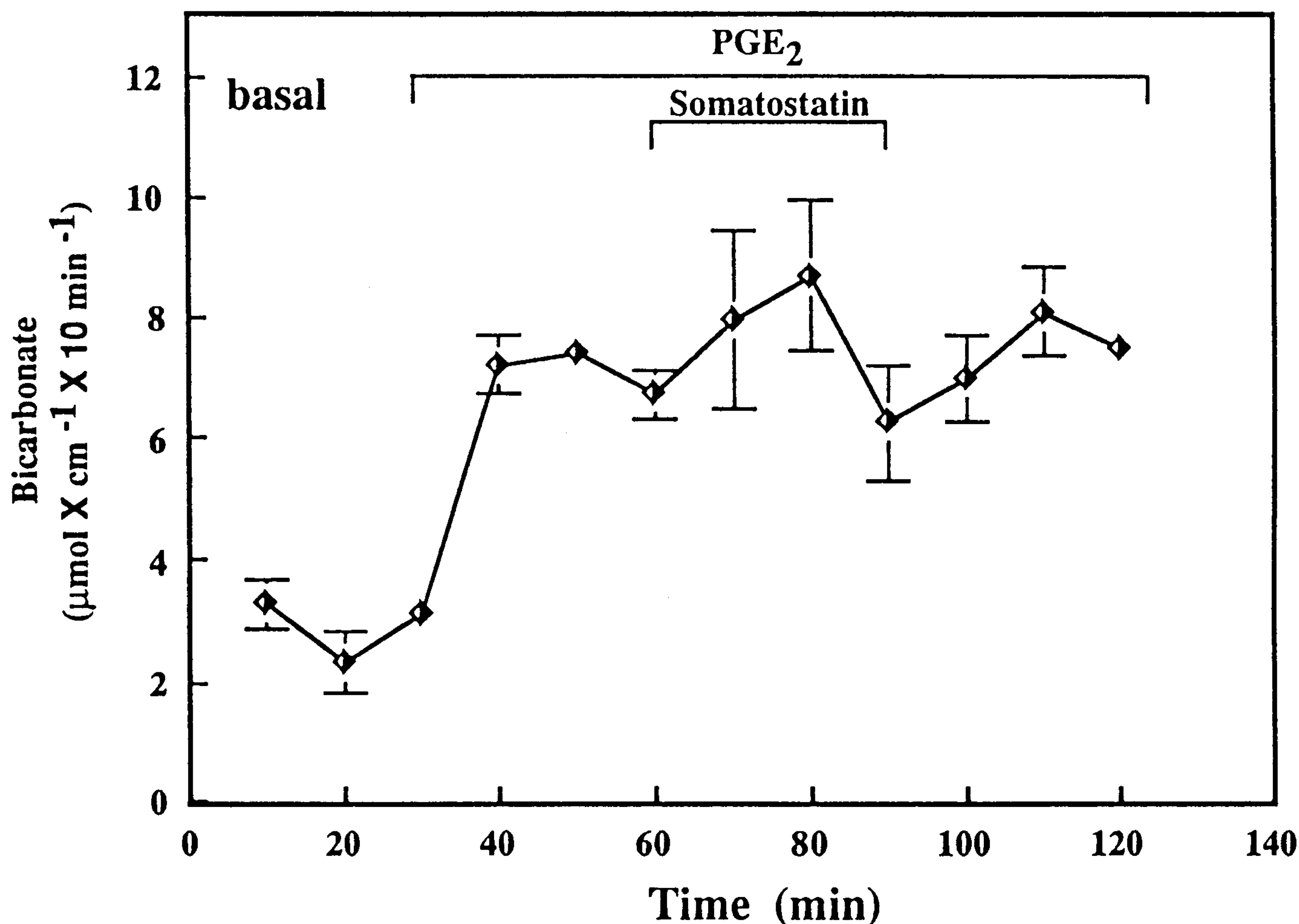


TABLE 13. Adenylate cyclase activity (measured as pmol cAMP.mg^{-1}.min^{-1}) in homogenized duodenal enterocytes treated with somatostatin and three agonists of duodenal HCO_3^- secretion

	Agonist alone	Agonist + somatostatin
Basal	9.4 ± 1.0	9.1 ± 1.7
Carbachol	9.9 ± 0.3	ND
VIP	21.8 ± 3.6*	20.7 ± 2.0*
PGE_2	30.9 ± 3.9*	28.0 ± 3.9*

Doses in mol.l^{-1}: somatostatin 10^{-6}, carbachol 10^{-3}, VIP 10^{-8}, PGE_2 10^{-7}. Vaues are expressed as mean ± SEM (n = 4). ND, not done. * P at least < 0.05 compared with basal.

EFFECT OF INHIBITION OF CARBONIC ANHYDRASE BY ACETAZOLAMIDE

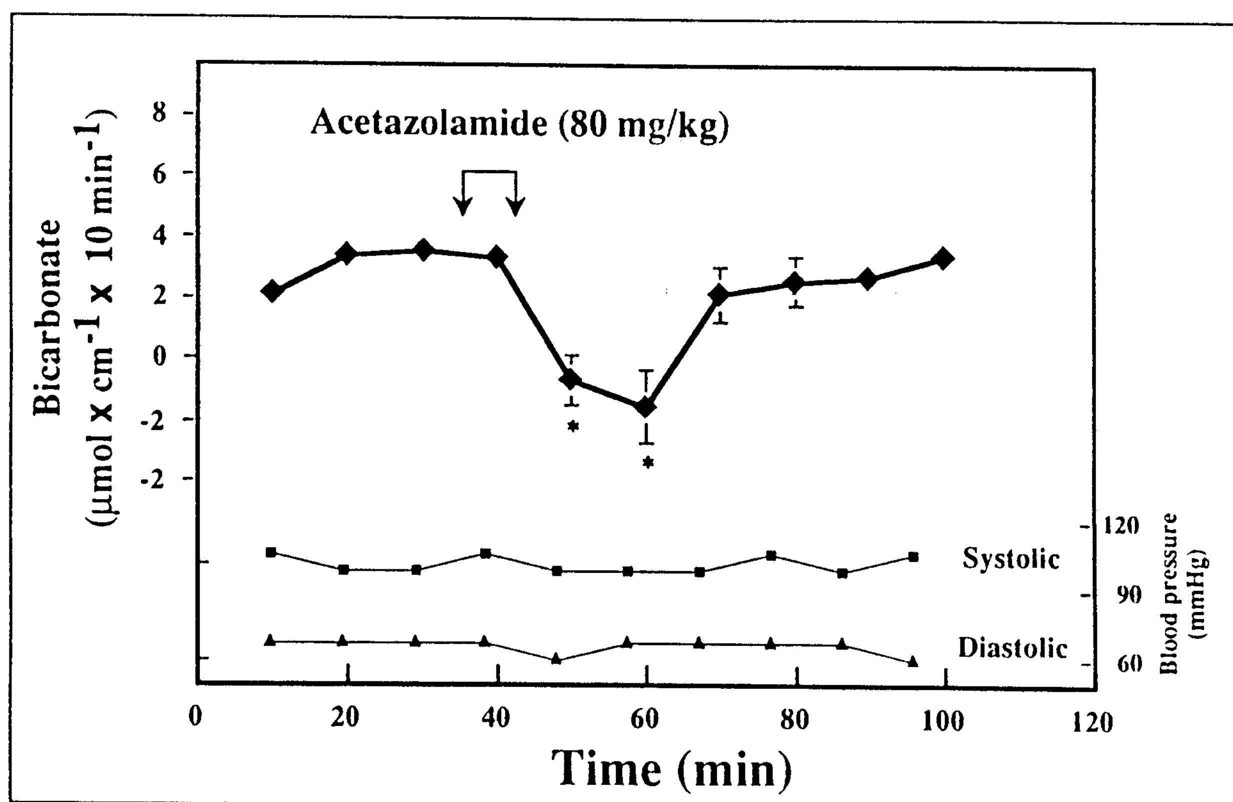
Effect of Acetazolamide on Basal Bicarbonate Secretion:

Basal bicarbonate output was shown to be 3.4 ± 0.2 $\mu mol.cm^{-1}.10\ min^{-1}$ (Fig. 15).

Following the intravenous infusion of acetazolamide ($3.6 \times 10^{-4}\ mol.kg^{-1}.5\ min^{-1}$, or $80\ mg.kg^{-1}.5\ min^{-1}$), the bicarbonate output decreased abruptly over a 20 minute period. The "negative values" of bicarbonate obtained indicated absorption of luminally perfused 24 mmol/l HCO_3^- along a concentration gradient (as stated, the blood of the anaesthetized guinea-pig was verified to contain 21 ± 1 mmol/l HCO_3^-).

Subsequently, there was a steady increase of bicarbonate secretion and a return to the steady basal level within 1 hour.

FIGURE 15. Effect of acetazolamide (80 mg.kg^{-1}.5 min^{-1} i.v.) on basal bicarbonate secretion by the isolated guinea-pig duodenum that was perfused with an isotonic solution containing 24 mol/l $NaHCO_3$. Net bicarbonate secretion is plotted in the upper portion of the graph and blood pressure in the lower portion. Values are mean ± SEM for n = 5 animals. SEM for blood pressure was < 5 mm Hg. *: $p < 0.05$ vs. basal.



Effect of Acetazolamide on VIP-Stimulated Bicarbonate Secretion:

VIP (10^{-8} mol.kg^{-1}.30 min^{-1}) i.v. significantly increased duodenal bicarbonate secretion from basal 3.4 ± 0.3 to maximum 10.8 ± 1.4 μmol.cm^{-1}.10 min^{-1} (Fig. 16).

During the combined infusion with acetazolamide (dose 3.6 x 10^{-4} mol.kg^{-1}.5 min^{-1}, or 80 mg.kg^{-1}.5 min^{-1}), the bicarbonate output was significantly reduced to 2.1 ± 1.9 μmol.cm^{-1}.10 min^{-1}.

In the post-acetazolamide period, when VIP was infused on its own again, the bicarbonate output rapidly returned to its former stimulated secretion rate of 9.8 ± 0.9 μmol. cm^{-1}.10 min^{-1}.

Effect of Acetazolamide on PGE_2-Stimulated Bicarbonate Secretion:

Acetazolamide (3.6 x 10^{-4} mol.kg^{-1}.5 min^{-1}) similarly reduced the bicarbonate output after stimulation with PGE_2 (10^{-6} mol.kg^{-1}.30 min^{-1}, Fig. 16).

Effect of Acetazolamide on Blood pH:

Blood gas and pH analysis was performed, to verify that there was no systemic acidosis, in at least one animal in each series of studies (i.e., effect of acetazolamide on basal bicarbonate secretion, and effect of acetazolamide on agonist stimulated bicarbonate secretion). There was no detectable acidosis in these animals. There were no changes of blood pressure in all the animals tested with acetazolamide.

Effect of Acetazolamide on Bicarbonate Secretion stimulated by Multiple Agonists:

Acetazolamide (3.6×10^{-4} $mol.kg^{-1}.5\ min^{-1}$) also reduced the bicarbonate output in response to stimulation with the agonists and secretory stimulants: VIP, prostaglandins, glucagon, carbachol, TPA and dBcAMP and dBcGMP (Table 14). These were all the agonists examined in this work.

These agonists alone stimulated bicarbonate secretion up to 275% of basal output. During the period of combined

FIGURE 16. Effect of acetazolamide (80 $mg.kg^{-1}.5\ min^{-1}$ i.v.) on VIP-and PGE_2 stimulated bicarbonate secretion by the guinea pig duodenum. The agonists VIP ($10^{-8}\ mol.kg^{-1}.30\ min^{-1}$) or PGE_2 ($10^{-6}\ mol.kg^{-1}.30\ min^{-1}$) were given i.v. for three consecutive periods of 30 minutes. The isolated duodenal loop was perfused with a solution containing 24 mmol/l HCO_3^- (and 130 mmol/l NaCl for isotonicity). Net bicarbonate secretion is plotted in the upper portion of the graph and blood pressure in the lower portion. Values are mean ± SEM, n = 4 animals. *: p < 0,05 vs. basal.

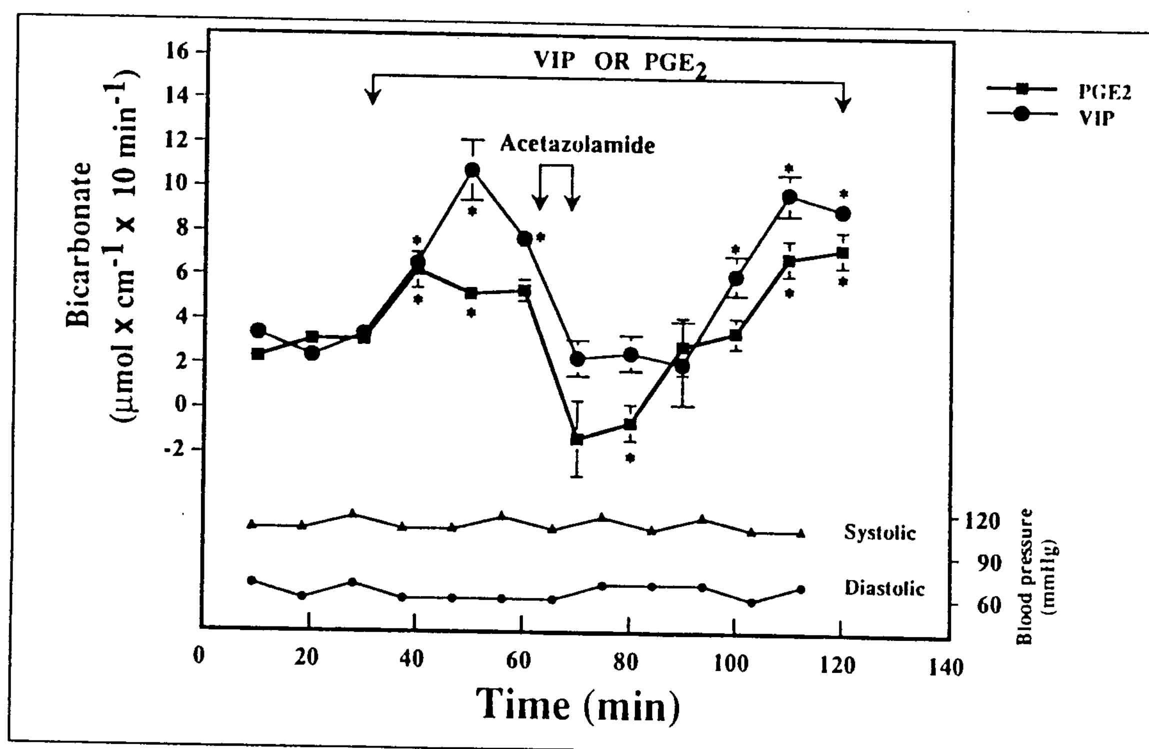


Table 14 Effect of acetazolamide infusion on stimulated bicarbonate secretion by the guinea-pig proximal duodenum. The agonists were infused iv (VIP, PGE_2, $PGF_{2\alpha}$, glucagon, carbachol, TPA) or intraduodenally (dBcAMP, dBcGMP) for three consecutive periods of 30 minutes, and acetazolamide (80 mg/kg iv for 5 min) was infused at the beginning of the second 30 minute period. Values are mean ± S.E.M. for n = 4 animals.

Agonist (mol/kg)	Basal	Agonist alone	Agonist + acetazolamide	Agonist alone
	HCO_3^- output (μmol x cm^{-1} x 30 min^{-1})			
VIP (10^{-8})	9.1 ± 0.5	25.1 ± 1.2[a]	7.0 ± 3.4[b]	25.0 ± 1.2[a]
PGE_2 (10^{-6})	8.5 ± 0.4	17.0 ± 1.2[a]	1.0 ± 2.5[ab]	17.7 ± 2.0[a]
$PGF_{2\alpha}$ (10^{-6})	10.4 ± 1.4	17.1 ± 2.3[a]	4.6 ± 1.3[ab]	17.6 ± 1.8[a]
Glucagon (10^{-7})	9.9±0.5	16.3 ± 0.8[a]	2.7 ± 0.6[ab]	20.1 ± 1.6[a]
Carbachol (10^{-8})	9.6 ± 0.7	20.2 ± 2.0[a]	0.5 ± 3.9[b]	20.5 ± 2.0[a]
TPA (10^{-7})	8.2 ± 0.4	22.5 ± 2.0[a]	-0.1 ± 3.0[ab]	24.3 ± 0.8[a]
dBcAMP (10^{-5})	6.9 ± 0.7	15.3 ± 2.0[a]	1.4 ± 3.1[b]	14.4 ± 2.8[a]
dBcGMP (10^{-5})	9.4 ± 0.7	12.9 ± 1.6[a]	1.3 ± 1.0[ab]	13.1 ± 1.6[a]

[a]: $p < 0.05$ vs. basal; [b]: $p < 0.05$ vs. agonist alone.

infusion of the respective agonists with acetazolamide, the bicarbonate output was significantly reduced, to 0 to 72 % of basal secretion.

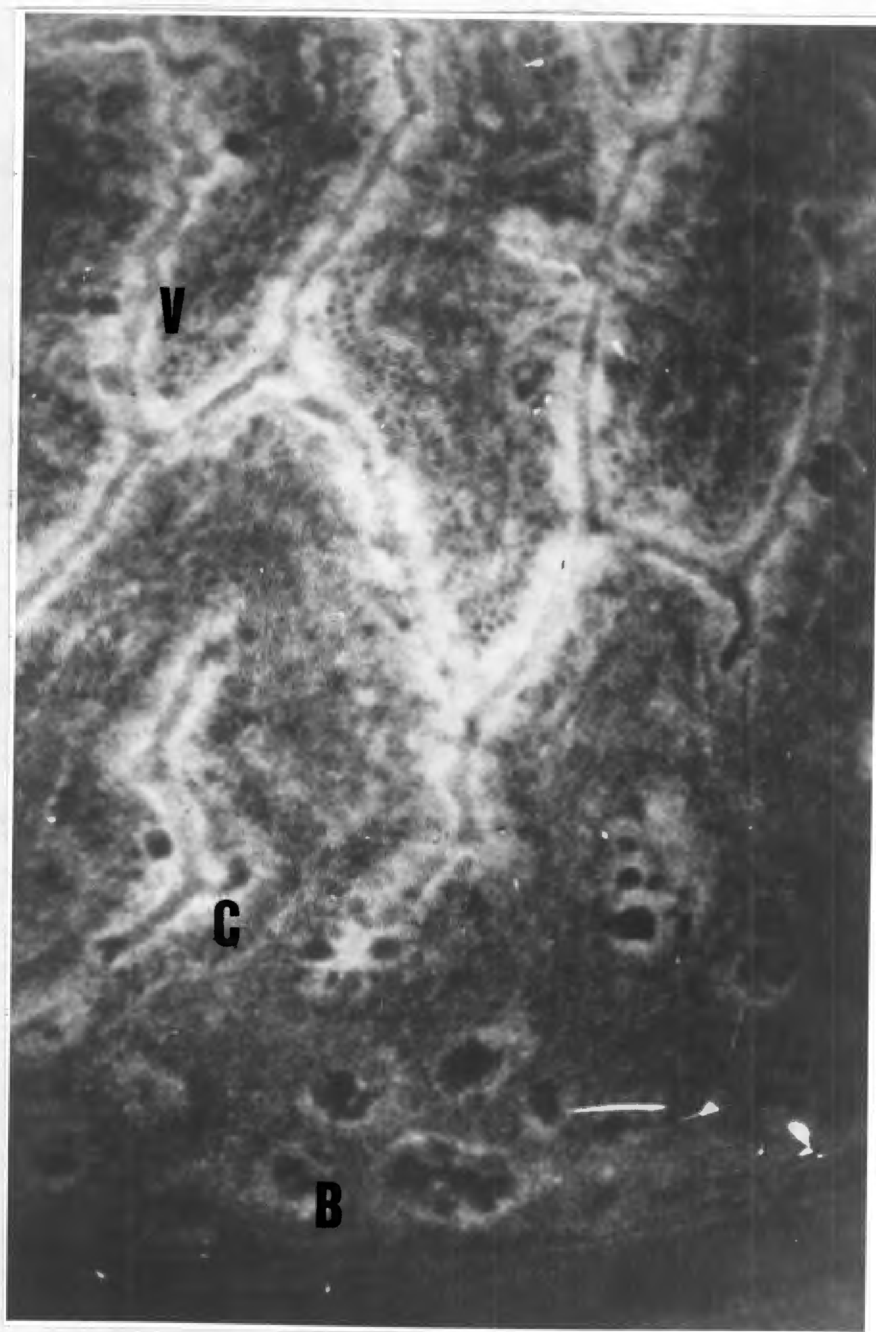
During subsequent infusion of each agonist alone, bicarbonate secretion returned to its former stimulated secretion rate.

Cellular Localization of Carbonic Anhydrase:

Carbonic anhydrase could be detected in all epithelial cells from the duodenal crypts up to the villi, except in the goblet cells (Fig. 17). The staining was present in the whole cytoplasm; no specific subcellular localization could be detected. The DNSA-fluorescence in Brunner's glands in the submucosa was faint (Fig. 17).

The specificity of this technique was demonstrated by the complete absence of fluorescence when incubating the sections with DNSA in the presence of 1 mmol/l acetazolamide. Of note, the DNSA-fluorescence of the stained duodenal specimens was lower than in sections from guinea pig stomach (the control, results not shown).

FIGURE 17. Carbonic anhydrase fluorescence in cryo-sections of the guinea-pig proximal duodenum. The section is slightly oblique, showing villi (V) and crypts (C). The whole epithelium from the crypts to the top of the villi shows a white fluorescence, which indicates the presence of the carbonic anhydrase-dimethylamino-naphthaline-5-sulfonamide complex in the mucosal cells. Brunner's glands (B) in the submucosa are scanty in this species and stain very faintly for carbonic anhydrase/DNSA complex.



DISCUSSION

ACTIVE SECRETION OF BICARBONATE: THE GUINEA-PIG MODEL

The first objective of this research was to set up a model where active bicarbonate secretion could be measured, that is to say, where there would not be diffusion of bicarbonate from the blood to the lumen.

This was achieved in the guinea-pig by perfusing the duodenal lumen with 24 mmol/l $NaHCO_3$ solution with added NaCl for isotonicity.

There has been considerable controversy in the literature regarding the amount of bicarbonate secreted actively by the duodenum, versus the quantity that diffuses passively from blood to lumen (Flemstrom 1987, Schiessel et al. 1984, Simson et al. 1981a). Most of the previous experiments on stimulation of duodenal bicarbonate secretion in intact animals were performed in the presence of a huge blood-to-lumen bicarbonate concentration gradient across the duodenal epithelium, while in vitro studies as in the Ussing chamber utilized a bicarbonate-free "luminal solution" and a bicarbonate-rich "serosal solution" (Simson et al. 1981a, Flemstrom & Garner 1982, Flemstrom et al. 1982a, Flemstrom et al. 1985, Garner et al. 1990).

The present guinea-pig model, based on Odes et al. (1990) and Heylings & Feldman (1988), was designed to

exclude passive diffusion of bicarbonate and permit the study of active transport of this anion into the duodenum. Since the concentration of bicarbonate in the luminal perfusate was about 3 mmol/l higher than that in the guinea-pig blood, uphill transport of bicarbonate against its chemical concentration gradient was examined.

While this model cannot exclude other gradients, such as electrical potential differences, or possible slight lumen-to-tissue diffusion of bicarbonate, passive diffusion of bicarbonate from the blood into the lumen is very unlikely. The present model appears to answer the need for an active secretion set-up in vivo.

Our finding, that duodenal bicarbonate secretion in the guinea-pig involves active transport processes, is in accordance with findings in man, where duodenal bicarbonate secretion could be stimulated by HCl and PGE_2 in the presence as well as the absence of a bicarbonate concentration gradient (Odes et al. 1990, Knutson et al. 1992).

It is now known that the duodenum secretes approximately equal amounts of bicarbonate, irrespective of whether the luminal perfusate contains isotonic NaCl (Flemstrom 1987), equimolar bicarbonate (Heylings & Feldman 1988, Odes et al. 1990) and hypermolar bicarbonate (Odes et al. 1990, and this study).

WHICH PART OF THE DUODENUM SECRETES BICARBONATE?

Since bicarbonate secretion decreases in the distal duodenum (Isenberg et al. 1983, Isenberg et al. 1986), the duodenal segment studied was sited as close to the bulb as possible in these studies. The bile duct enters the duodenum of the guinea-pig immediately distal to the pylorus, and the perfused segment began at that point. The area studied therefore corresponded closely to the part of the duodenum which in man is afflicted by peptic ulceration.

The proximal portion of the guinea-pig duodenum contains small numbers of Brunner's glands (Fig. 2). Our intention was to study mucosal bicarbonate secretion. Fortunately, there is quite convincing evidence that Brunner's glands do not secrete bicarbonate. Secretin, which stimulates Brunner's glands, did not increase basal duodenal bicarbonate secretion in another study (Hogan & Isenberg 1991) and in our study. The distal mammalian (Flemstrom 1987) and proximal amphibian (Simson et al. 1982) duodenum, which are devoid of any Brunner's glands, do secrete bicarbonate. The bicarbonate secretion attributed to Brunner's glands in a previous study by Kirkegaard et al. (1984) was likely derived from the duodenal mucosa.

We conclude from these observations that in our experiments we tested mucosal secretion of bicarbonate. However, to finally resolve this controversial issue, it will be necessary to devise the methodology whereby viable

duodenal mucosa, stripped of all Brunner's glands, can be mounted and tested for bicarbonate secretion in Ussing-type chambers.

AGONISTS OF BICARBONATE SECRETION

Selection of Agonists for these Studies:

The agonists of duodenal bicarbonate secretion, that were examined in these studies, were selected according to reports in the literature (Table 1), with the addition of TPA, which was not tested before.

PGE_2 and VIP were selected for study, since they appear to be the chief mediators of acid-induced bicarbonate secretion. Physiological doses of hydrochloric acid entering the duodenum release both mucosal VIP and PGE_2 into the blood and lumen, increasing duodenal bicarbonate secretion in a variety of preparations in vivo and in vitro (Simson et al. 1981b, Flemstrom et al. 1982, Flemstrom et al. 1985, Odes et al. 1990, Smedfors et al. 1990). Prostanoid synthesis was demonstrated in the duodenal submucosa, while degradation occurred predominantly in the epithelial cells (Smith et al. 1987). Furthermore, in histochemical studies, VIP-containing nerve fibres were demonstrated in the lamina propria of the mucosa, forming networks around the crypts and extending up in the cores of the villi (Larsson et al.

1976). These findings indicate the participation of both PGE_2 and VIP in the regulation of the secretory status of the mucosal epithelium. Therefore, these agonists were given special emphasis in the present study.

Carbachol was studied because of the profound effect of vagal stimulation on bicarbonate secretion (Ballesteros et al. 1991, Granstam et al. 1987, Konturek & Thor 1987, Lenz et al. 1989, Nylander et al. 1987, Safsten & Flemstrom 1986).

Glucagon and secretin, which belong to the VIP "family" of peptides, were also tested. The importance of glucagon as an agonist of bicarbonate secretion was uncertain. Glucagon inhibited basal, secretin- and CCK-stimulated pancreatic bicarbonate secretion (Owyang 1991). Secretin, however, is the major agonist of pancreatic bicarbonate secretion (Schulz 1987).

Since PGE_2, VIP, secretin and glucagon act via the adenylate cyclase and protein kinase A cascade, dBcAMP and theophylline were selected for study. Carbachol however has a different action involving protein kinase C. TPA and $PGF_{2\alpha}$ act much like carbachol. Therefore, dBcGMP was also studied. It should be clear however that these intracellular cascades are probably interconnected for many ligands. For example, secretin stimulates cyclic AMP and inositol triphosphate production in rat pancreatic acinar tissue by two fully independent mechanisms (Trimble et al. 1987).

Dose-Response Data with Selected Agonists:

We have demonstrated that dBcAMP, VIP, PGE_2, carbachol, TPA, theophylline, dBcGMP, PGF_{2a} and glucagon stimulate duodenal bicarbonate secretion against a bicarbonate gradient, this representing active secretion. Secretin was not effective as a stimulant (Fig. 3).

The results show clearly that both the adenylate cyclase/cAMP/PKA cascade (stimulated by dBcAMP, theophylline, VIP, glucagon and PGE_2), and the inositol phospholipid/PKC cascade (stimulated by dBcGMP, carbachol, TPA, and PGF_{2a}) were involved in secretion of bicarbonate by the duodenum.

These agents differ in their potency to stimulate bicarbonate secretion (although it should be noted that dBcAMP was given intraduodenally, and the others i.v.), but this was not dependant on the identity of the intracellular messengers.

The most potent agonists, on a molar basis, were dBcAMP, VIP, PGE_2 and carbachol (Table 2). TPA appeared to be equally potent, but a direct comparison is impossible, since TPA was tested only in the dose of 10^{-7} mol/kg. Theophylline was intermediate in its effectiveness. Glucagon and dBcGMP were weaker stimulants of bicarbonate secretion. Thus, these results represent the first direct comparison of the various agents, using the same animal and experimental protocol, and demonstrate that the agonists can increase bicarbonate

secretion in the face of a concentration gradient, so that diffusion of bicarbonate is not being measured.

Yao et al. (1993) showed in rabbit duodenum, maintained in a chamber in vitro, that VIP was much more potent (about 2 log units) than PGE_2, theophylline and dBcAMP, in that order. Rapid in vivo degradation of VIP would explain the "greater" potency of VIP in Yao's work compared to our study in the guinea-pig. Also, Yao's luminal perfusate was unbuffered, while bicarbonate (25 mmol/l, pH 7.4) was present on the serosal side and diffusion of bicarbonate was not prevented. Of note, the rabbit duodenum requires pre-treatment with indomethacin to depress prostaglandin synthesis and reduce the high resting rate of bicarbonate secretion.

Effect of Combinations of Agonists:

These studies (Tables 3 to 9), designed to pin-point which internal messengers activate bicarbonate secretion, yielded most interesting data. Agonists (in submaximal doses) utilizing the same, or different, intracellular messengers were infused together. The results show that combining agonists using different intracellular pathways leads to increased (roughly summative) secretion of bicarbonate. Combining agonists using identical intracellular pathways, however, leads to decreased secretion of bicarbonate.

The first result was anticipated, since the doses of the agonists in each pair would on their own produce submaximal production of duodenal bicarbonate, and further secretion of bicarbonate was therefore possible.

The reason for the second result, the lack of an additive effect when two agents acting on the same intracellular messenger were combined, is not clear. Several lines of reasoning were considered.

There appeared to be no extraintestinal causative factor, such as, for, example, a reduction of systemic blood pressure when VIP and PGE_2 were given together; however, intraduodenal blood flow was not measured, and might have been altered by these potent "vasoactive" agents, thus affecting generation of bicarbonate. A more likely possibility relates to interactions at the receptors at the duodenal enterocytes. Could there have been interactions at the cell membrane, where one agonist (example: VIP) prevented another agonist (like: PGE_2) from activating its receptor, so that bicarbonate secretion decreased? Yet another, related explanation refers to "negative" interactions of second messenger proteins within the duodenal enterocytes. The true reason is not known, and awaits further experimental study.

It is interesting that a very similar phenomenon was noted by Yao et al. (1991), who studied stimulated bicarbonate secretion in the rabbit duodenum mounted in the Ussing chamber. Yao et al. found that when dBcAMP was

combined with VIP or PGE_2, the bicarbonate secretion was less than when dBcAMP was used alone as a stimulant. Further, the combination of VIP and PGE_2 stimulated less bicarbonate secretion than either agonist on its own. These data are similar to our own and suggest immediately that tissue blood flow cannot be the explanation. As stated above, the answer must lie in receptor or post-receptor phenomena in the duodenal enterocytes.

The data presented here, and those of Yao et al. (1991), could have distinct physiological consequences, since they clearly imply that bicarbonate secretion is reduced when certain compounds (utilizing identical intercellular messengers) stimulate the duodenum in unison. This could be important especially in the case HCl-induced physiological bicarbonate secretion, which, as indicated above, is mediated jointly by VIP and PGE_2.

Cyclic AMP as the Second Messenger of PGE_2 and VIP:

The demonstration that dBcAMP stimulates duodenal bicarbonate secretion in vitro (Simson et al. 1981b, Garner et al. 1990, Yao et al. 1991) implies participation of cAMP as a second messenger in stimulated bicarbonate secretion. This however still had to be proved. For this reason, we tried to mimic the effects of PGE_2 and VIP on duodenal bicarbonate secretion by using intraduodenal dBcAMP, and we measured cAMP production in duodenal enterocytes exposed to

VIP, PGE_2 and other agonists (in a collaborative study with Dr. R Reimer, Germany).

VIP and PGE_2, which concentration-dependently increased active duodenal bicarbonate secretion in our in vivo model, stimulated adenylate cyclase of homogenates prepared from isolated duodenal epithelial cells. By contrast, secretin and $PGF_{2\alpha}$, showing no or little effect on bicarbonate secretion, stimulated adenylate cyclase to a considerably lesser extent than VIP and PGE_2 (Reimer et al. in press). The increased intracellular cAMP levels in duodenal epithelial cell homogenates were next followed by an increase in the activity of protein kinase A. Timewise, these increases preceded the rise in duodenal bicarbonate secretion as was detected in the guinea-pig perfused duodenal segment in vivo. These events followed each other in a way that is likely for coupled processes; at the time the preceding stimulus reaches its maximum, the following event shows its steepest slope (Fig. 6). These observations fulfill the criteria of Sutherland (1968) for establishing that cAMP mediates intestinal secretion.

Our conclusion is that VIP and PGE_2 stimulate active bicarbonate secretion in the guinea-pig duodenum via increases in intracellular cAMP and protein kinase A.

This theory is supported by several studies. Garner et al. (1990) stimulated duodenal bicarbonate secretion, in vivo and in vitro, with the phosphodiesterase inhibitors, theophylline and IBMX, which raise intracellular cAMP

levels. Simson et al. (1981b) stimulated amphibian duodenal bicarbonate secretion in vitro by theophylline, and Yao et al. (1991) found a similar effect of dBcAMP and theophylline in the rabbit duodenum in vitro. VIP and PGE_2 are known to act via receptors coupled to stimulation of adenylate cyclase in isolated enterocytes in the rat (Nakaki et al. 1983) and guinea-pig (Binder et al. 1980); this has now been shown in the human as well (Smith et al. 1990). By contrast, the high doses of secretin required for stimulation of adenylate cyclase in vitro (Reimer et al. in press) indicate a pharmacological response, and are consistent with the lack of effect of this hormone on bicarbonate secretion in the guinea-pig in vivo (Fig. 4). Glucagon did not stimulate adenylate cyclase; it appears to be a weak stimulant of duodenal bicarbonate secretion and it could be acting via another mediator (as shown for carbachol, see above).

Receptors for VIP in the Guinea-Pig Duodenal Enterocytes:

Stimulation of active transport processes necessarily involves the activation of specific transport systems via intracellular signalling mechanisms.

We have recently reported the presence of receptors for VIP (Reimer et al. 1993b) on duodenal epithelial cells, which are coupled to stimulation of adenylate cyclase. In that study, VIP stimulated adenylate cyclase in homogenates

of duodenal epithelial cells with a more than 1000-fold higher potency than secretin.

These results agree well with the results of receptor binding studies by Binder et al. (1980), where secretin was shown to be 1000-fold less potent than VIP in inhibiting binding of radiolabeled VIP in the guinea-pig small intestine. This argues for the presence of a VIP-receptor on duodenal epithelial cells.

These in vitro data of Binder et al. (1980) correspond well with the physiological response in the present study, where the neuropeptide VIP (10^{-9} mol/kg) stimulated bicarbonate secretion 3-fold in the perfused guinea-pig duodenal segment (Fig. 3 and 4), while the structurally related hormone secretin, in the same dose (10^{-9} mol/kg), was ineffective in stimulation of duodenal bicarbonate secretion (note that in this dose, secretin was a powerful agonist of pancreatic bicarbonate secretion).

We can now hypothesize that binding of VIP to its receptors, which are located on basolateral membranes (Dharmsathaphorn et al. 1983), causes activation of adenylate cyclase, also located in basolateral membranes (Murer et al. 1976), which then leads to the formation of cAMP in duodenal enterocytes. This then is the process whereby VIP induces active duodenal bicarbonate secretion in the guinea-pig.

Receptors for Prostaglandins in the Guinea-Pig Duodenal Enterocytes:

Recently, our group published that this prostanoid receptor is of the EP_2-subtype (Reimer et al. 1992). PGE_2 binds with high affinity to this type of receptor (Coleman et al. 1990). Specific prostanoid binding was shown in rat small intestinal preparations by Wu-Wang et al. (1989). Coupling of the prostanoid receptor to adenylate cyclase was demonstrated by Smith et al. (1987).

$PGF_{2\alpha}$ is known to have a very low affinity for EP_2-receptors. This is confirmed by our finding that stimulation by $PGF_{2\alpha}$ of adenylate cyclase was very small. Our finding that $PGF_{2\alpha}$ can stimulate duodenal bicarbonate secretion, although to a lesser extent than PGE_2, indicates the involvement of other effects than increases in intracellular cAMP levels in $PGF_{2\alpha}$-stimulated duodenal bicarbonate secretion, and implies that other cellular pathways are working.

The Link with Protein Kinase A:

In all systems in which the effect of cyclic AMP is understood, adenylate cyclase-cyclic AMP induced activation of proteins results from changes in regulation of phosphorylation/dephosphorylation. Cyclic AMP activates cAMP-dependent protein kinases by binding to the regulatory

subunit and release of the catalytic subunit (Gettys & Corbin 1989). We have reported (Reimer et al. 1991) that upon stimulation of duodenal cells with VIP and PGE_2, the soluble (cytosolic) PKA activity increased, while the total amount of PKA in the particulate fraction decreased, indicating a translocation from the membranes to the cytosol.

The cAMP effect on intestinal transport is likely to occur directly at the brush border membrane. Phosphorylation of specific membrane proteins by cAMP-dependent protein kinases was shown for the rat small intestine (de Jonge 1976, Shlatz et al. 1978). In addition, Dunk et al. (1989) showed that incubation of rat duodenal brush border membrane vesicles with cAMP and ATP stimulated Cl^-/HCO_3^- exchange by a cAMP-dependent protein kinase. Since both cAMP and the catalytic subunit of protein kinase A are soluble, activation of basolateral membrane adenylate cyclase can lead to activation of cAMP-dependent proteins throughout the cell. Although the highest PKA activity was recovered in the cytosolic fraction, large amounts were found in the particulate fraction. This is in agreement with findings in membrane preparations of intestinal epithelial cells, where cAMP-dependent protein kinase activity could be detected in basolateral and brush border membranes (Shlatz et al. 1978). In addition, histochemical studies showed high cAMP binding to basolateral membranes, indicating the presence of intracellular, membrane-bound receptors for cAMP (Ong et al.

1975). Other polarized cells like proximal and collecting duct epithelial cells also showed a high amount of cAMP-dependent protein kinases in plasma membranes (Kinne et al. 1975). This could be the basis for a spatial model explaining how VIP and PGE_2 activate duodenal bicarbonate secretion.

CHOLINERGIC REGULATORY MECHANISMS

In our detailed studies of the cholinergic regulation of duodenal bicarbonate secretion in the guinea-pig, there were three main findings:

(i) The cholinergic agonist carbachol stimulates bicarbonate secretion by the m-cholinoceptor pathway.

(ii) Carbachol-stimulated bicarbonate secretion is not a direct effect but proceeds via the neuropeptide hormone VIP, and the lack of demonstrable receptors for carbachol on isolated duodenal enterocytes is consistent with this observation.

(iii) The cholinergic pathway is dependent on the presence of intracellular Ca^{2+}.

Muscarinic Effects:

While central vagal stimulation increases duodenal bicarbonate secretion (Ballesteros et al. 1991, Flemstrom & Jedstedt 1989, Konturek & Thor 1987, Lenz & Forquignon 1990, Lenz et al. 1989), carbachol, bethanechol and atropine have shown inconsistent and weak effects on basal and stimulated duodenal bicarbonate secretion (Ballesteros et al. 1991, Granstam et al. 1987, Hogan & Isenberg 1991, Konturek & Thor 1987, Lenz et al. 1989, Smedfors & Johansson 1986, Takeuchi et al. 1989, Takeuchi et al. 1990). This could have been the result of species differences, but has given the impression that cholinergic stimulation is less important than that of prostaglandins and VIP.

We differ from these reports by demonstrating here that carbachol is a potent stimulant of bicarbonate secretion in the guinea-pig, and that atropine and pirenzepine inhibit basal and carbachol-stimulated bicarbonate secretion.

On a weight basis, the relative inhibitory potency of parenteral atropine to pirenzepine on exocrine glands is 1:10 (Jaup 1981). The doses of pirenzepine and atropine that produced similar inhibition of carbachol-stimulated bicarbonate secretion were in the same ratio.

While this suggests that the cholinergic pathway for bicarbonate secretion is of the m_1-cholinoceptor type, additional studies with specific m_2- and m_3-cholinoceptor antagonists are required to finalize this issue. A previous

report that carbachol requires an m_2-cholinoceptor pathway, was based only on the failure of pirenzepine to inhibit carbachol-stimulated bicarbonate secretion (Takeuchi et al. 1990), and appears to be premature.

The present results with pirenzepine, which inhibited bicarbonate secretion, cannot be reconciled with previous observations in the rat that this agent stimulated duodenal bicarbonate secretion (Safsten & Flemstrom 1986, Takeuchi et al. 1990). The reason for this discrepancy is not apparent.

Nicotinic Effects:

Since hexamethonium also reduced carbachol-stimulated duodenal bicarbonate secretion, a role for a non-muscarinic cholinergic transmitter is possible.

Hexamethonium also inhibited the effect of vagal stimulation on duodenal bicarbonate secretion in rat (Fandriks et al. 1991), while atropine only partially inhibited the bicarbonate response to sham feeding in man (Ballesteros et al. 1991). Hexamethonium, but not atropine, inhibited the prostaglandin E_2-mediated bicarbonate secretion which followed duodenal acidification (Smedfors et al. 1990).

The substantial hypotension following the injection of hexamethonium, however, could itself depress the secretion of bicarbonate, via stimulation of the $alpha_2$ adrenoceptors (Fandriks et al. 1991). Indeed, we observed a brisk return

of carbachol-stimulated duodenal bicarbonate secretion once the blood pressure rose. Therefore, our results with hexamethonium, as well as those of earlier workers, do not allow of any firm conclusion regarding an n-cholinoceptor transmitter in duodenal bicarbonate secretion.

VIP as a Cholinergic Mediator of Bicarbonate Secretion:

VIP is an agonist of duodenal bicarbonate secretion (Flemstrom et al. 1985, Algazi et al. 1989, Wolosin et al. 1989). Our observations with the VIP-antagonist support the notion that the cholinergic pathway to bicarbonate secretion is largely mediated via VIP.

Of note, pirenzepine had no effect on VIP-mediated bicarbonate secretion. Unlike Smedfors et al. (1987), we did show partial atropine-inhibition of VIP-stimulated bicarbonate secretion; the mechanism of this action is not clear. We have reported that specific VIP receptors exist on duodenal mucosal cells (Reimer et al. 1993b); therefore, the "presence" of another mediator after VIP would mean that an unidentified intracellular agent (protein) is involved, or that there are different cell populations among the duodenal enterocytes. This issue needs to be resolved.

Smedfors et al. 1987 and Algazi et al. (1989) found that HCl-stimulated duodenal bicarbonate secretion is mediated by VIP. We showed that basal bicarbonate output too requires some VIP activity. [D-p-Cl-Phe^{6},Leu^{17}] VIP as well

as atropine blocked the stimulatory effect of intracerebral somatostatin on rat duodenal bicarbonate secretion (Lenz & Forquignon 1990), which is in keeping with our finding that VIP acts as a cholinergic neurotransmitter in the duodenum. By comparison, prostaglandin-stimulated duodenal bicarbonate secretion is independent of the action of VIP (Algazi et al. 1989), and not affected by atropine (Smedfors et al. 1990) or by pirenzepine, as we have shown here.

Role of Intracellular Calcium:

We studied the role of calcium in stimulus-secretion coupling associated with carbachol-induced bicarbonate secretion.

Verapamil abolished carbachol-stimulated bicarbonate secretion, as was described previously (Takeuchi et al. 1990), as well as basal bicarbonate secretion. A similar effect on bicarbonate secretion was detected with the more specific calcium channel blocker, nifedipine. This shows that Ca^{2+} is required for cholinergically stimulated bicarbonate production in the duodenum.

This notion is further supported by our finding that A23187 dose-dependently stimulated basal bicarbonate secretion, and modestly but significantly augmented the effect of carbachol when these two agents were combined.

A23187 also stimulated amphibian duodenal bicarbonate secretion in vitro (Simson et al. 1982).

The site of action of A23187 appears to be proximal to the duodenal enterocytes which carry VIP receptors, since the VIP antagonist blocked A23187-stimulated duodenal bicarbonate secretion. It appears that calcium is required for the cholinergic release of VIP, the mediator of carbachol-induced bicarbonate secretion, and that verapamil and nifedipine may act to prevent release of VIP after carbachol stimulation.

Whether cholinergic stimulation releases mediators other than VIP, as well is not known, but the lack of effect of indomethacin on carbachol-stimulated bicarbonate secretion in the present and a previous study (Takeuchi et al. 1990) does indicate that the Ca^{2+}-mediated pathway is independent of endogenous prostaglandin synthesis.

Taken together, the results show that carbachol-stimulated duodenal bicarbonate secretion is mediated by m-cholinoceptors, vasoactive intestinal peptide and calcium. Calcium has a role in mediating bicarbonate secretion both in the basal state (resting vagal tone) and after cholinergic stimulation.

Receptors for Carbachol in the Guinea-Pig Duodenal Enterocytes:

In our studies, as reported by Reimer et al. (1993a), there was a virtual absence of ^{3}H-N-methylscopolamine binding and a failure of the intracellular Ca^{2+} concentration to increase in response to carbachol stimulation of isolated duodenal enterocytes. This indicates that the duodenal enterocytes contain very few m-cholinoceptors and that carbachol stimulation of duodenal bicarbonate secretion requires an intermediate messenger. We have shown that this is VIP.

Postulated Cholinergic Pathway:

Our postulates of the pathway involved in the cholinergic stimulation of duodenal bicarbonate secretion are shown schematically in Fig. 18.

The uncertainties in this pathway relate to the role, if any, of n-cholinoceptors, and the meaning of inhibition of VIP by atropine (the "post-VIP" ligand). These issues require further investigation.

However, it should be understood that the adenylate cyclase-protein kinase A cascade is not the only intracellular messenger of bicarbonate secretion, even if it

appears to mediate cholinergic secretion (from the role described herein for VIP). We have found a clear response to stimulation with TPA, demonstrating that protein kinase C is involved in bicarbonate secretion as well (Odes et al. 1992). Furthermore, atropine partly inhibited the action of VIP. The pathways involved are certainly more complex than is presently known.

FIGURE 18. Proposed pathway of the cholinergic regulation of guinea-pig duodenal bicarbonate secretion. The cholinergic stimulus activates the m_1-cholinoceptors (m_1), and possibly also the m_2-cholinoceptors (m_2), located on VIP-secreting cells situated in close proximity to vagal nerve terminals in the duodenum. The role of the n-cholinoceptor (n) is not clear, given the hypotensive effect of hexamethonium in our experiments. VIP production or secretion requires calcium entry via nifedipine-sensitive channels, since the VIP- antagonist [D-p-Cl_Phe^6,Leu^{17}]-VIP inhibited the effect of the calcium ionophore A23187. VIP in turn activates VIP-receptors presumed to be present on duodenal mucosal cells that secrete bicarbonate. The mechanism of atropine-inhibition of VIP-stimulated bicarbonate secretion requires elucidation; pirenzepine had no such effect. Note that the bold arrows indicate stimulation and the thin arrows indicate inhibition of secretion.

ROLE OF SOMATOSTATIN IN DUODENAL BICARBONATE SECRETION

The present experiments examined the influence of somatostatin-14 on guinea-pig duodenal bicarbonate secretion.

Effect on Basal and Stimulated Bicarbonate Secretion:

The depressant action of somatostatin on basal duodenal bicarbonate secretion in our study is consistent with the general inhibitory effects of this hormone in the gastrointestinal system (Yamada & Chiba 1989).

Certain earlier studies are at variance with our data. The lack of effect of somatostatin on basal bicarbonate secretion by frog duodenal mucosa in vitro (Flemstrom et al. 1982a) could possibly be explained by the already low basal bicarbonate secretion rate in that model. The failure of i.v. somatostatin-14 to reduce basal duodenal bicarbonate secretion in the conscious rat (Lenz & Forquignon 1990) is however difficult to explain since the same dose (10^{-11} $mol.kg^{-1}$) was effective in the present experiments (Fig. 12). Somatostatin-14 inhibited basal bicarbonate output in the anaesthetized rat by decreasing the volume of duodenal secretion (Kirkegaard et al. 1984). Of note, somatostatin-28 is not an agonist of duodenal bicarbonate secretion (Lenz & Forquignon 1990).

It is of interest that somatostatin inhibited both carbachol-stimulated and VIP-stimulated duodenal bicarbonate secretion. Cassuto et al. (1983) showed that in the process of intestinal secretion, a VIP-ergic neuron is situated distal to the cholinergic neuron. Lenz et al. (1989) reported that TRH-induced vagal stimulation of duodenal bicarbonate secretion is mediated by cholinergic pathways and VIP. We have demonstrated (the present study, and Odes et al. in press) that carbachol, at least in part, exerts its effect on duodenal bicarbonate secretion via the release of VIP. In the guinea pig-ileum, somatostatin hyperpolarizes almost exclusively neurons immunoreactive for VIP, and in this way inhibits the release of VIP (Cooke 1989). Therefore, somatostatin may inhibit carbachol-stimulated bicarbonate secretion by an action related to VIP. This postulated effect of somatostatin on VIP could also explain the inhibitory effect of somatostatin on basal bicarbonate secretion, a process that is largely under vagal control (Ballesteros et al. 1991). Kirkegaard et al. (1984) too showed that somatostatin decreased VIP-stimulated bicarbonate secretion in rat duodenum in vivo.

VIP and PGE_2 both stimulated adenylate cyclase activity in the present study. We have reported that VIP and PGE_2 act via distinct receptors on duodenal epithelial cells, increasing intracellular cAMP by activation of adenylate cyclase (Reimer et al. in press, Reimer et al. 1993b). In the amphibian and mammalian duodenum, an increase in

intracellular cAMP by phosphodiesterase-inhibitors led to increased bicarbonate secretion (Garner et al. 1990). While somatostatin inhibited VIP-stimulated bicarbonate secretion, it did not inhibit adenylate cyclase. Furthermore, PGE_2-stimulated bicarbonate secretion was not affected. These findings, taken together, appear to indicate that somatostatin does not act directly at the duodenal enterocytes.

The inhibitory effect of somatostatin on VIP-stimulated bicarbonate secretion is best explained by postulating that somatostatin prevents the release by VIP of another mediator (possibly cholinergic) which then stimulates bicarbonate secretion. In support of this hypothesis is our finding that VIP-stimulated duodenal bicarbonate secretion is partly atropine-sensitive (Odes et al. in press).

Our present understanding of the role of somatostatin-14 in regulating duodenal mucosal bicarbonate secretion is illustrated in Fig. 19.

Activity of Somatostatin in Duodenal Enterocytes:

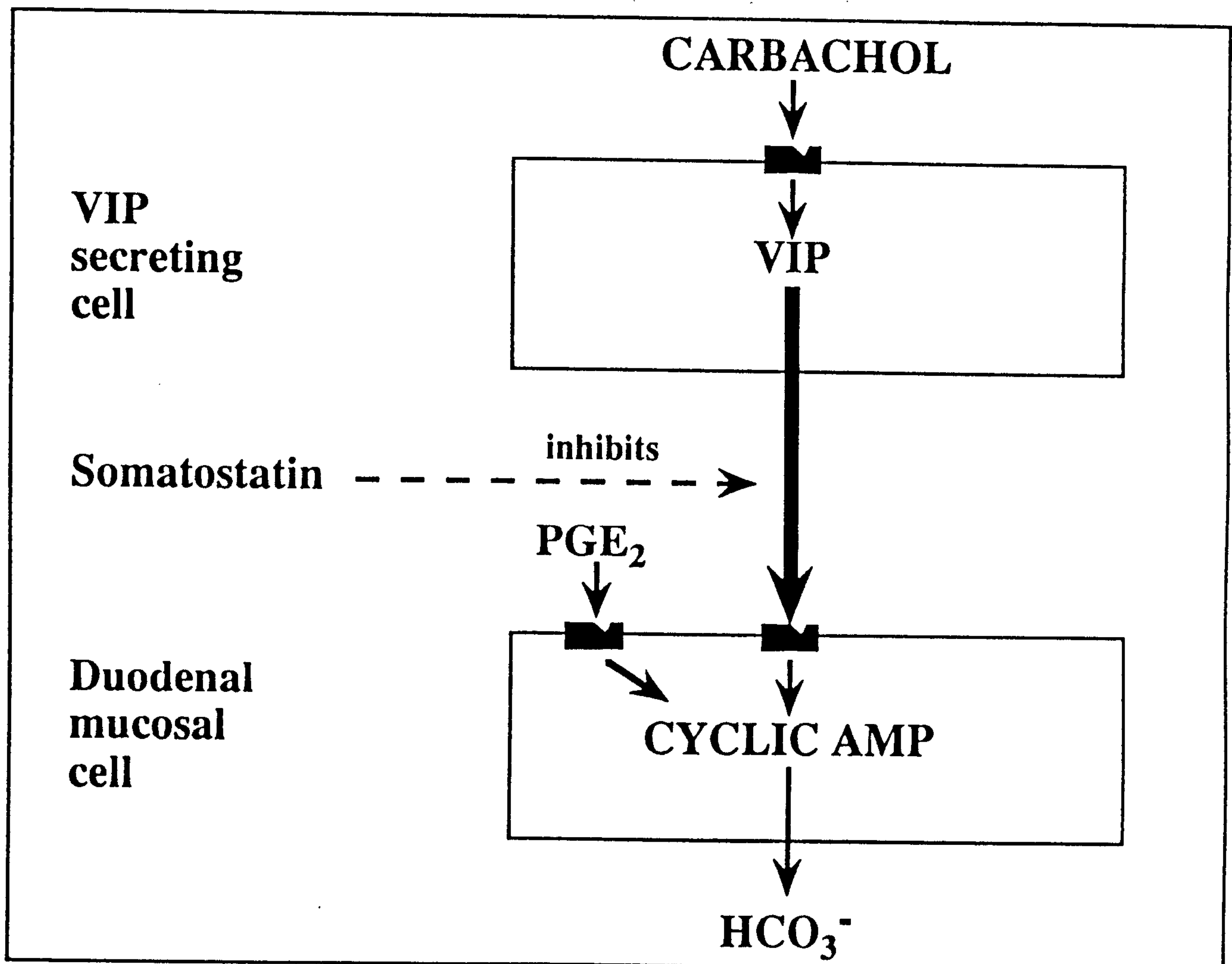
Molecular cloning has revealed the presence of four high-affinity somatostatin receptors (SSTR) acting on specific intracellular signalling systems. This includes the SSTR1 receptor in the upper small intestine, whose effector system is apparently not coupled to adenylate cyclase (Bell & Reisine 1993).

This is in keeping with the lack of effect of somatostatin on duodenal enterocyte cAMP production in the present study. It is not likely that this phenomenon is the result of damage caused to the somatostatin receptor in the cell homogenization process, since the receptors of VIP and PGE_2 were functional in our preparations of duodenal enterocytes. It remains possible that intracellular mediators other than cAMP are coupled to the somatostatin receptor in the duodenum. However, since we did not detect cholinoceptors on guinea-pig duodenal enterocytes (Reimer, 1993), the effect of somatostatin on protein kinase C and intracellular Ca^{2+} was not examined in the present study.

Immunity of PGE_2 to the Effects of Somatostatin:

Cholinergic stimulation to the upper gastrointestinal tract has multiple physiological effects. There is release of acid in the stomach (Isenberg et al. 1991), and release of PGE_2 (Bukhave et al. 1990), VIP (Smedfors et al. 1987) and somatostatin (Fujimiya et al. 1992) in the duodenum. PGE_2 amplifies duodenal bicarbonate secretion independently of VIP (Algazi et al. 1989). VIP, as shown, is not effective in the presence of somatostatin. Of note, PGE_2 levels may be

FIGURE 19. Postulated mechanism of action of somatostatin on duodenal HCO_3^- secretion. Carbachol, which did not stimulate mucosal adenylate cyclase, stimulates duodenal HCO_3^- secretion via VIP. It is postulated that VIP releases an unidentified, possibly cholinergic, mediator that is inhibited by somatostatin at a site proximal to the duodenal enterocyte, since cyclic AMP production is unaltered by somatostatin. PGE_2 stimulates cAMP formation in the duodenal enterocytes and this action is not affected by somatostatin.



increased in the inflamed duodenum. Therefore, it is conjectured that the "immunity" of PGE_2 to the effects of somatostatin-14, as shown by the present data (Fig. 14), may constitute a protective mechanism in the acidified duodenum. The action of PGE_2 in stimulating bicarbonate secretion, an action insensitive to somatostatin, is an important duodenal defence mechanism; thus, antiinflammatory drugs like indomethacin, which inhibit PGE_2 synthesis, are ulcerogenic.

In summary, the present study has shown a role, best described as "selective", for somatostatin-14 in the regulation of duodenal bicarbonate secretion. The precise pathways involved require further investigation.

ROLE OF CARBONIC ANHYDRASE IN DUODENAL BICARBONATE SECRETION

These studies have shown that carbonic anhydrase is required for active bicarbonate transport under basal conditions, and for active bicarbonate secretion stimulated by dBcAMP, dBcGMP, PGE_2, $PGF_{2\alpha}$, TPA, glucagon, VIP and carbachol. It was shown that carbonic anhydrase is distributed equally in crypt and villus cells in the guinea-pig duodenum.

Duodenal Carbonic Anhydrase and Bicarbonate Secretion:

The duodenum contains one tenth the carbonic anhydrase of the gastric antrum in the rat; in both organs the enzyme

is located largely in the cytosol (Stiel et al. 1984, and our own study).

Studies of the function of carbonic anhydrase in duodenal bicarbonate secretion have yielded conflicting data.

Thus, acetazolamide had a variable effect on bicarbonate secretion by the bullfrog duodenum in vitro, with a high dose being required to produce mild inhibition (Flemstrom 1980, Simson et al. 1981a). This is consistent with the notion that low rates of bicarbonate secretion, as in the bullfrog in vitro, do not require carbonic anhydrase. In the rat, the drug did not reduce basal bicarbonate secretion (Flemstrom & Kivilaakso 1983, Takeuchi et al. 1986); the effect on PGE_2- and $PGF_{2\alpha}$ - stimulated secretion was either inhibitory (Flemstrom & Kivilaakso 1983, Isenberg et al. 1983) or negligible (Takeuchi et al. 1986). In the rabbit, where resting bicarbonate secretion is high (the result of a high basal prostaglandin synthesis), acetazolamide reduced basal bicarbonate secretion (Holm et al. 1990). Like the rabbit, the guinea pig has a high basal bicarbonate secretion rate too (Flemstrom et al. 1982a), which is due to a high basal prostaglandin synthesis; therefore, in the present study the animals were pre-treated with indomethacin to reduce this background stimulation.

Carbonic anhydrase seemingly cannot be stimulated by agonists. We have found (Odes & Reimer, preliminary

observations), that incubation of isolated duodenal cells with three potent agonists of bicarbonate secretion, PGE_2, VIP or carbachol did not show any increase in the activity of carbonic anhydrase enzyme.

Source of Bicarbonate:

The mechanisms of transport of bicarbonate to the gut lumen are not clearly understood. There is evidence that bicarbonate can be transported to the duodenal lumen both transcellularly and paracellularly, by serosal CO_2- and HCO_3^--dependent processes (Flemstrom 1980, Simson et al. 1981a); similar observations were made in the distal colon (Rho et al. 1991). In the frog duodenum in vitro there was considerable paracellular diffusion of CO_2 which accounted for 40% of bicarbonate secretion, and little endogenous generation of HCO_3^- by carbonic anhydrase (Simson et al. 1981a). In the rabbit duodenum exposed to intraluminal acid, bicarbonate transport appeared to be passive (Vattay et al. 1988, Vattay et al. 1989). By contrast, acetazolamide decreased basal, PGE_2 and HCl-stimulated bicarbonate secretion in the human duodenum (Knutson et al. 1992).

Unlike previous workers using rodents, we have shown significant decreases of basal and stimulated bicarbonate secretion in the presence of acetazolamide, regardless of the absolute secretory rate. This suggests that the secreted

bicarbonate, at least in part, is generated intracellularly by carbonic anhydrase-mediated hydration of CO_2.

Our finding that acetazolamide reduced bicarbonate secretion in response to a variety of agonists implies that carbonic anhydrase, providing a source of bicarbonate, functions as a common step in stimulus secretion coupling leading to bicarbonate secretion. Carbonic anhydrase may be linked to a luminal Cl^-/HCO_3^- exchanger present in isolated duodenocytes (Flemstrom & Garner 1982, Gleeson 1992).

Other Functions of Carbonic Anhydrase:

An important consideration in interpreting the action of acetazolamide in laboratory experiments relates to the ability of this drug to cause acidosis in small animals. The dose of acetazolamide employed in the present study however is reported to be non-toxic (Lonnerholm et al. 1989). This dose did not cause cardiac and respiratory side-effects or acidosis in our animals, as shown by the lack of respiratory changes and the stability of blood pressure and pulse tracings and blood pH (where tested) during the infusion of acetazolamide. The absorption of luminal bicarbonate during acetazolamide treatment occurred along its concentration gradient, possibly by a paracellular pathway, and was not caused by acidosis; we observed a similar phenomenon during

inhibition of duodenal bicarbonate secretion with somatostatin in the current experiments.

The dose of acetazolamide used here (80 mg/kg) calculates to about 3.4 x 10^{-4} mol/l in the animal body water, sufficient to produce over 99% inhibition of carbonic anhydrase (Lonnerholm et al. 1989). In addition to inhibiting carbonic anhydrase, acetazolamide in this dose could also have inhibited a bicarbonate export mechanism at the cell membrane of duodenal epithelial cells. The present data do not allow of a conclusion regarding this possibility. Hence, the precise function of carbonic anhydrase in the duodenum requires further investigation. In particular, the relationship between carbonic anhydrase and cell membrane exporters or transporters of bicarbonate remains to be determined.

Site of Bicarbonate Secretion along the Duodenal Villus-Crypt Axis:

The identity of the duodenal epithelial cells responsible for bicarbonate secretion is unknown. It is thought that, in the entire small intestine, the crypt is secretory and the villus absorptive (Donowitz & Welsh 1987). Since the duodenal villi are most exposed to acidic duodenal chyme, bicarbonate secretion by the villi would be an appropriate defense.

The present finding, that carbonic anhydrase is located equally in the villi and crypts, may indicate that both these areas are sites of bicarbonate secretion. These results differ from a previous study by Lonnerholm (1976) in guinea-pig jejunum, where staining for carbonic anhydrase could be detected in a small number of epithelial cells only. These differences are probably due to the less sensitive cobalt precipitation technique used by Lonnerholm (1976). An immunohistochemical study in the rat showed that carbonic anhydrase II, the dominant acetazolamide-sensitive isoenzyme in the duodenum, was confined to the villus epithelium (Lonnerholm 1989), and this suggested that alkaline secretion is of villus origin. This was confirmed in the rat jejunum, where it was shown that net secretion in the small bowel is probably not confined to the crypts and may also occur from villus epithelium (Stewart & Turnberg 1989). We did not determine which isoenzyme of carbonic anhydrase was present in the duodenum of the guinea pig. In rat duodenum, carbonic anhydrase II predominates while carbonic anhydrase I is scanty (Lonnerholm 1976).

Clinical Relevance of Carbonic Anhydrase in Bicarbonate Secretion:

The clinical importance of carbonic anhydrase in bicarbonate secretion is not yet properly defined, and has been little studied.

In normal man, acetazolamide (0.5 g/day orally for 2 days) was shown to reduce PGE_2-stimulated and acid-stimulated bicarbonate secretion (Knutson et al. 1992). Further studies are needed in this area.

SUMMARY: REGULATION OF DUODENAL BICARBONATE SECRETION

The guinea-pig duodenal model studied here contained many interacting tissues and cells, but had the advantage that bicarbonate secretion could be measured directly at all times. By complementing these studies with various in vitro experiments, a partially comprehensive picture of the control of active duodenal bicarbonate secretion is beginning to emerge.

It is clear that dBcAMP, VIP, PGE_2 and theophylline are among the chief agonists of duodenal bicarbonate secretion in the guinea-pig. All these agonists act through adenylate cyclase. Correlation with in vitro data shows that VIP and PGE_2 act directly via receptors on the duodenal enterocytes,

activating adenylate cyclase and protein kinase A in sequence to initiate bicarbonate secretion. Theophylline and dBcAMP act internally in the duodenal enterocytes.

Glucagon is a weak agonist of bicarbonate secretion, not altering duodenal cAMP production; its physiological importance is in doubt. Of note, secretin is not an agonist of bicarbonate secretion, although it does have receptors in the duodenum.

Carbachol is a potent agonist of active bicarbonate secretion and acts in the m-cholinoceptor pathway. It does not act directly on duodenal epithelial cells, but stimulates duodenal bicarbonate secretion by releasing VIP. Consistent with this finding is the observation that carbachol itself has few receptors on duodenal enterocytes. It can thus be said that carbachol, working via VIP, also utilizes the adenylate cyclase cascade. The cholinergic pathway requires intracellular Ca^{2+}, perhaps for the release of VIP. In contrast to the knowledge obtained about the muscarinic pathway, the role of the nicotinic pathway in bicarbonate secretion remains uncertain, owing to the hypotensive action of hexamethonium.

There is evidence that the inositol phospholipid and protein kinase C cascade are involved in bicarbonate secretion, possibly to a lesser extent, since tetradecanoyl-phorbol-acetate and $PGF_{2\alpha}$ were agonists of bicarbonate secretion.

Somatostatin selectively suppresses carbachol-stimulated and VIP-stimulated duodenal bicarbonate secretion, but not PGE_2-stimulated bicarbonate secretion. Receptors for somatostatin coupled to adenylate cyclase could not be detected on isolated duodenal enterocytes, which strengthens the hypothesis that carbachol does not act directly on these epithelial cells, but via a second transmitter, which is VIP. The immunity of PGE_2 to the actions of somatostatin appears to be a protective mechanism. Cholinergic stimulation, as during gastric acid secretion, releases VIP, PGE_2 and somatostatin. VIP may be less effective in stimulating bicarbonate production under these conditions, but PGE_2 will continue to be an agonist of bicarbonate secretion, offering protection to the duodenal mucosa which is bathed by HCl.

Carbonic anhydrase activity is necessary for secretion of bicarbonate. Inhibition of this enzyme by acetazolamide decreases basal duodenal bicarbonate secretion and stops the stimulation by diverse agonists. Carbonic anhydrase likely serves as a common final step in the generation of bicarbonate in duodenal enterocytes. Carbonic anhydrase is located in the cytoplasm of cells in the villus as well as the crypt cells, implying that bicarbonate secretion occurs along the length of the villus and crypt.

In summary, the present research has shown direct stimulation of duodenal bicarbonate secretion by VIP, which participates also in the m-cholinergic pathway, and by PGE_2.

Adenylate cyclase and protein kinase A appear to be the intracellular messengers with the primary function of initiating duodenal bicarbonate secretion. However, there is convincing evidence that the inositol phospholipid and protein kinase C cascade also activates this secretion. Somatostatin selectively stops duodenal bicarbonate secretion. Carbonic anhydrase activity in the crypt and villus is required as the final common step in bicarbonate production.

The findings of this research provide a basis for further, more comprehensive studies of bicarbonate secretion in the duodenum of man in health and disease.

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