

## Acid Secretion and the H,K ATPase of Stomach

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The regulation of acid secretion was clarified by the development of H<sub>2</sub>-receptor antagonists in the 1970s. It appears that gastrin and acetylcholine exert their effects on acid secretion mainly by stimulation of histamine release from the enterochromaffin-like (ECL) cell of the fundic gastric mucosa. The isolated ECL cell of rat gastric mucosa responds to gastrin/cholecystokinin (CCK), acetylcholine, and epinephrine with histamine release and to somatostatin and R- $\alpha$ -methyl histamine by inhibition of histamine release. Histamine and acetylcholine stimulate the parietal cell by elevation of cAMP or [Ca]<sub>i</sub> by activation of H<sub>2</sub> or M<sub>3</sub> receptors, respectively. These independent pathways converge to activate the gastric acid pump, the H<sup>+</sup>,K<sup>+</sup> ATPase. Activation is a function of the association of the ATPase with a potassium chloride transport pathway that occurs in the membrane of the secretory canaliculus of the parietal cell. Hence the secretory canaliculus is the site of acid secretion, the acid being pumped into the lumen of the canaliculus. The pump is composed of two subunits, a large catalytic and a smaller glycosylated protein. This final step of acid secretion has become the target of drugs also designed to inhibit acid secretion. The target domain of the benzimidazole class of acid pump inhibitors is the extracytoplasmic domain of the pump that is secreting acid, and the target amino acids are the cysteines present in this domain. The secondary structure of the pump can be analyzed by determining trypsin-sensitive bonds in intact, cytoplasmic-side-out vesicles of the ATPase, and it has been shown that the  $\alpha$  subunit has at least eight membrane-spanning segments. Omeprazole, the first acid pump inhibitor, forms a disulfide bond with cysteines in the extracytoplasmic loop between the fifth and sixth membrane-spanning segment and to a cysteine in the extracytoplasmic loop between the seventh and eighth segments, preventing phosphorylation of the pump by ATP. As a result of the effective and long-lasting inhibition of acid secretion by the acid pump inhibitor, superior clinical results have been found in all forms of acid-related disease.

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### INTRODUCTION

Acid secretion by the stomach is a universal attribute of all vertebrates. Its function is to aid digestion of food by allowing peptic activity, to kill prey that is ingested live, and to help sterilize gastric contents. Hippocrates was the first to speculate as to how the stomach carried out digestion; his idea was that the stomach cooked the food. He had not, however, seen the constituents of haggis. The Italian physiologist, Spallanzani, is credited with the concept that the gastric juice dissolved,

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*Abbreviations:* CCK: cholecystokinin ECL: Enterochromaffin-like (cells) EGF: epidermal growth factor FITC: fluorescein isothiocyanate NSAID: non-steroidal anti-inflammatory drug(s)

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rather than cooked, the food. With the beginning of modern inorganic chemistry, it was then shown, by the Frenchman, Prout, that the secretion of the stomach was muriatic acid, HCl. The cellular origin of gastric acid was defined in 1895, by Golgi, the Italian microscopist, who described the expansion of the secretory canaliculus of the stimulated parietal cell. This expansion was illustrated with remarkable accuracy, using a camera lucida. The concept of active transport was first enunciated in 1905, by Overton, but then remained largely ignored until the work of Boyle and Conway in frog muscle. They were the first to show that muscle extruded sodium and accumulated potassium. Their experiment was first to cool frog muscle and show sodium accumulation and then, with rewarming, to show extrusion of sodium and accumulation of potassium [1]. Hypotheses as to the mechanism of acid secretion by the parietal cell were already in place in the 1920s, when a redox mechanism was conceived, following the discovery of the redox enzymes in mitochondria by Keilin and Hartree. This concept postulated a separation of electrons and hydrogen across the acid secretory membrane, following oxidation of a reduced substrate; the acid was secreted and the electrons were accepted by a cytoplasmic oxidant [2].

This concept antedated by 30 years Peter Mitchell's recognition that the mitochondrial membrane contains redox-driven electrogenic proton pumps and that it is the collapse of the electrochemical gradient of  $H^+$  across the mitochondrial ATP synthetase that is responsible for the synthesis of ATP. This chemiosmotic mechanism was one of the great insights of modern biology. That Peter Mitchell was suffering from ulcer disease at the time of his discovery was not a coincidence [3].

The discovery of ATP by Engelhardt in 1938 led in the 1960s to the idea that ATP, not redox reactions, was the source of energy needed for acid secretion by the parietal cell. The sodium pump was described in 1957 by Jens Skou, and was shown to exchange  $3Na$  for  $2K$ , thus explaining the Boyle and Conway experiments [4]. The close relationship of the sodium pump to the acid pump of the stomach was not realized until the 1970s [5,6,7]. It remained for molecular biology to show that the gastric acid pump had significant homology to both the  $\alpha$  and  $\beta$  subunits of the sodium pump [8,9].

Preliminary observations on the regulation of acid secretion were described in patients with gastric fistulas, but Pavlov described in detail the cephalic phase of secretory regulation in his classic studies in dogs at the beginning of the twentieth century. The presence of the humoral factor, gastrin, was suggested by Edkins in the early years of this century also, and the discovery of acetylcholine by Loewi and of histamine by Dale in the 1920s set the stage for another of the controversies surrounding gastric function; namely, whether gastrin, acetylcholine, or, in particular, histamine were the major stimulants of acid secretion.

Clinical interest in acid secretion relates to peptic ulcer disease. The adage "no acid, no ulcer" has resulted in a variety of therapies designed to reduce acid secretion. These therapies can be divided into surgical and medical methods. The early surgical efforts at reduction of acid secretion involved total or partial gastric resection, developed by Billroth in Vienna. This surgery thus removed the acid-secreting part of the stomach. These procedures were improved upon by vagotomy and then highly selective vagotomy designed to inhibit stimulation of gastric acid secretion rather than to remove the acid-secreting gastric fundus. Most recently, laparoscopic, highly selective vagotomy is being introduced into surgical practice, to minimize trauma to the patient.

Medical therapy has depended on advances in understanding of gastric physiology and biochemistry, and the targets were the receptor first and then the pump. The first drug recognized to inhibit acid secretion was extract of belladonna, deadly nightshade. The active principle in this extract is atropine, the non-selective, but potent, muscarinic antagonist, which inhibits vagal stimulation of acid secretion. Prostaglandins of the E<sub>2</sub> subtype were, some 70 years later, also shown to inhibit gastric acid secretion. Both of these classes of drugs have side effects that limit their use, such as dry mouth or blurred vision in the case of atropine, diarrhea in the case of prostaglandins. The synthesis and the description of the actions of the H<sub>2</sub> histamine receptor antagonists by Black, Duncan, Durant, Ganellin, and Parsons in 1971 [10] heralded a new era in the medical treatment of peptic diseases, whereby acid secretion could be inhibited at the receptor level with drugs that had few, if any, side effects. The global action of the H<sub>2</sub> antagonists on acid secretion stimulated by histamine or gastrin resolved the controversy regarding pathways of stimulation, showing that the action of gastrin was mediated by the release of histamine.

The variability of response to H<sub>2</sub>-receptor antagonists, and the need for more profound inhibition in, for example, gastroesophageal reflux disease, resulted in the development of another class of drug able to inhibit acid secretion, the acid pump inhibitor class [11]. This short review will discuss some aspects of regulation of acid secretion and some properties of the pump. It is probably correct to say that the advances in medical therapy in the past two decades have made surgery the exception, rather than the rule, in acid-related disease. Understanding of the mechanisms of the parietal cell has allowed these therapies to evolve.

## REGULATION OF ACID SECRETION

### *Stimulation of Acid Secretion*

In most species, acid secretion varies from a basal to a stimulated state, depending on the digestive requirements at the time secretion is measured. Stimulation of the parietal cell is both centrally mediated by vagal parasympathetic fibers and peripherally mediated by release of histamine from the enterochromaffin-like (ECL) cell, acetylcholine from the vagus, and gastrin from the G cell of the antrum.

Gastrin is released from the G cell due to stimulation by aromatic amino acids. Presumably there are receptors on the G cell that bind amino acids, which, in the bound state, produce typical activation of second messengers such as [Ca]<sub>i</sub> or cAMP. There are also neuronal pathways that stimulate gastrin release from the ECL cell. Somatostatin released from the D cell of antral mucosa inhibits gastrin release, as does luminal acid. Whether the luminal acid acts directly on the G cell or only indirectly by stimulating release of somatostatin is not clear, although current thinking suggests that the latter is the predominant pathway.

The controversy about pathways of stimulation began with the sequencing of gastrin by Gregory and his collaborators [12]. That advance in the early 1960s emphasized the role of this peptide as radioimmunoassays became available. With these methods of analysis, it was shown that meals elevate serum gastrin, and that this elevation was sufficient to account for the stimulation of acid secretion. Hence gastrin appeared to be the major component of the peripheral regulatory mechanism. Neutralizing antibodies for gastrin inhibit meal-induced acid secretion, but

such data show that gastrin is essential, not necessarily sufficient, for stimulation of the parietal cell by food in the stomach [13].

The synthesis and the description of the action of  $H_2$  antagonists [10] altered both the treatment of acid disease and the understanding of peripheral regulation of secretion. It was shown, contrary to the expectations of many, that these receptor antagonists were extremely effective inhibitors of acid secretion during pentagastrin stimulation. Indeed, they were able even to inhibit partially secretion due to vagal stimulation. Since neither gastrin nor acetylcholine bind to the  $H_2$ -receptor, it is clear that histamine must intervene in the pathway of stimulation initiated either by gastrin or acetylcholine. This theory justified the idea that histamine was the final common pathway for stimulation of acid secretion, a concept perhaps more correct than the promotion of gastrin as the final mediator. Nevertheless, this unifying concept was still an oversimplification. For stimulation of acid secretion, there are parallel pathways that converge only partially by the release of histamine.

### *The ECL Cell*

The search for the cell responsible for paracrine secretion of histamine was only undertaken after the action of  $H_2$ -receptor antagonists was defined. Mast cells were long known to contain histamine and to be present in the mucosa, so at first it was thought that mast cells were the origin of the relevant histamine. It was shown, however, that mast cells do not respond to gastrin and that, at least in the rat, the mast cells were few and in the wrong place in the fundic mucosa. A cell, the enterochromaffin-like cell, was then considered to be the cell that releases histamine in response to gastric secretagogues [14].

This cell represents between 0.5 and 1 percent of the cell population of the normal stomach. It is recognized by its size (10  $\mu\text{m}$ ), specific staining properties, and a characteristic morphology. The cytoplasm includes numerous vacuoles containing eccentric osmophilic granules. Only recently have the properties of this cell type become accessible to direct experimentation. Figure 1 shows two cells present close to parietal cells in a scanning electron micrograph of an isolated rabbit gastric gland which, by size and position, are likely to be ECL cells. Purification of ECL cells from rat can be followed quite easily by using the characteristic uptake of acridine orange by the histamine-containing granules of this 10  $\mu\text{m}$  diameter cell (Fig. 2).

Purification of the ECL cell of the rat [15] has allowed the direct demonstration that gastrin cholecystokinin (CCK), and acetylcholine all release histamine from this cell. Gastrin stimulation is accompanied by a change in intracellular calcium characteristic of epithelial cells, namely, a transient increase followed by steady-state elevation. The latter is due to calcium entry, and this plateau phase is the important determinant of histamine release. A typical calcium signal is illustrated in Fig. 3, where both release from calcium stores and calcium entry are shown as stimulating histamine release. In addition, there is a cAMP-dependent release of histamine, but the primary physiological secretagogue responsible for elevation of cAMP in the cell is not known. Epinephrine, however, stimulates histamine release in the rat ECL-cell preparation, and this release is blocked by the  $\beta$  antagonist, propranolol, and hence in this species there is coupling of histamine release to activation of a  $\beta$  adrenergic receptor [15]. Perhaps this class of receptor is responsible for stress ulceration in the rat and also, perhaps, in man.

The histamine is stored in acidic granules. The storage is dependent on the activity

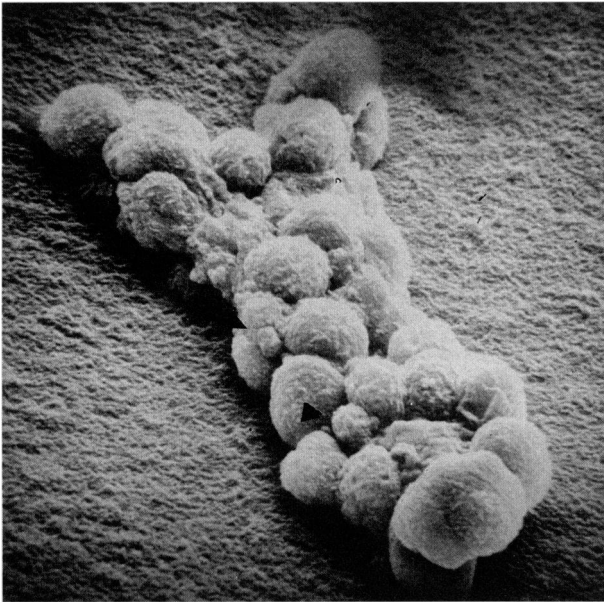


FIG. 1. A scanning electron micrograph of a rabbit gastric gland, showing the large parietal cells. Between two clusters of parietal cell, a small cell with the diameter expected of an ECL cell is indicated by an arrow.

of a V-type ATP-driven proton pump, and histamine uptake is probably coupled to  $H^+$ /histamine countertransport. Histamine is released by exocytosis, stimulated by the ligands categorized above. The histamine is likely released in proximity to parietal cells in the rabbit, since chief cells in this species have an adrenergic, but not a histamine, receptor [16]. Perhaps in rabbit, epinephrine is also stored in the ECL cell. It appears also that the ECL cell has a histamine  $H_3$ -receptor subtype, since  $H_3$  antagonists elevate and  $H_3$  agonists suppress gastrin-stimulated histamine release [15]. The action of these  $H_3$  compounds on cat gastric mucosal acid secretion can be explained by their action on the ECL cell [17].

The model of the function of the ECL cell in acid secretion that is emerging is

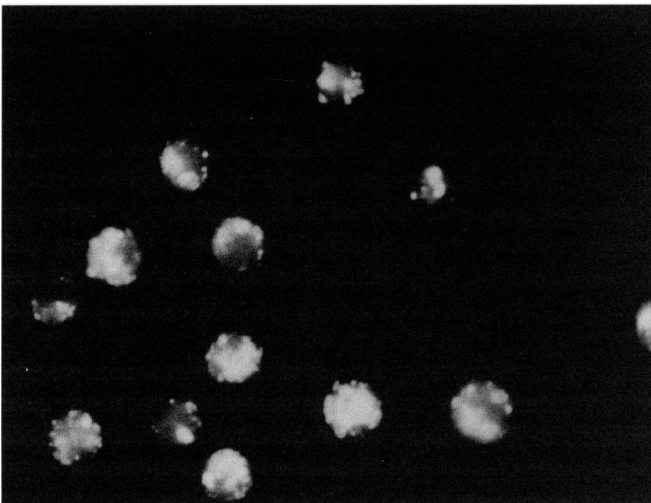


FIG. 2. A sample of the purified rat ECL-cell preparation stained with acridine orange [15]. The dye has been taken up and concentrated, and the metachromatic shift of the dye changes the fluorescence from green to red.

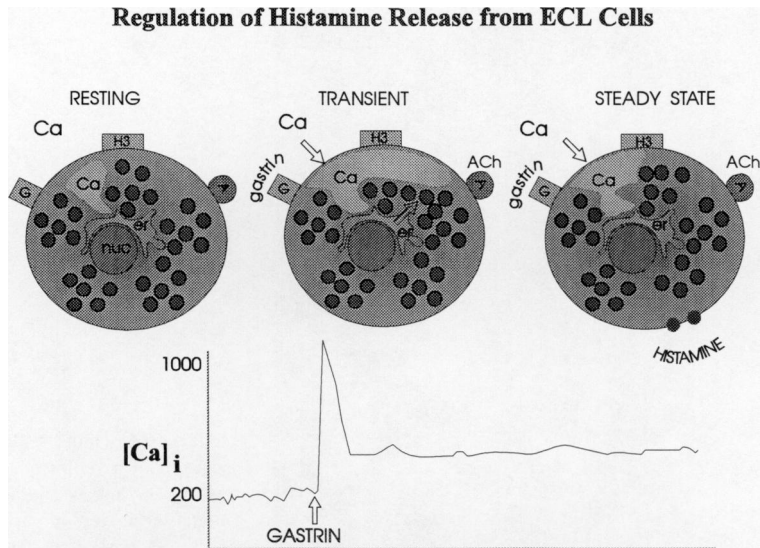


FIG. 3. A model of the calcium release and calcium uptake in ECL cells induced by gastrin. Shown is an intracellular calcium pool and a resting calcium level of a cell in short-term culture. Addition of gastrin stimulates calcium release and calcium entry, and calcium entry is maintained for as long as the hormone is present.

shown in Fig. 4, but it must be regarded in some aspects as tentative. The target of the histamine released from the ECL cell is the  $H_2$ -receptor on the parietal cell.

#### *The Parietal Cell*

The morphology of this cell is also quite characteristic. It is a large, 20  $\mu\text{m}$  conical cell, containing a large number of mitochondria, relatively sparse Golgi, and a well-developed cytoplasmic membrane system containing the acid pump, called the tubulovesicular complex and, at rest, a narrow infolding of the plasma membrane, the secretory canaliculus, as described by Golgi. When the cell is stimulated, there is loss of the tubulovesicles and a large expansion of this structure, the secretory canaliculus, which becomes lined with slender microvilli, now containing the acid pump (Fig. 5). In Fig. 5, the gastric H,K ATPase was stained by an  $\alpha$  subunit-specific antibody and illustrates the change both in morphology and the location of the H,K ATPase [18].

There are three known classes of activating receptors on the parietal cell, the histamine, the  $H_2$ -receptor, the muscarinic  $M_3$  receptor, and the gastrin receptor. These receptors appear to have different efficacies in terms of activation of acid secretion. There is also an epidermal growth factor (EGF) receptor, which inhibits histamine-stimulated acid secretion. A prostaglandin  $EP_3$  receptor subtype also inhibits histamine-stimulated acid secretion. A recent paper has claimed that the site of action of these secretagogues may be the immune system in the lamina propria of the stomach [19], but this concept is unable to explain the direct effects of stimuli seen in single isolated parietal cells, using video imaging [21,23], nor the effects of stimuli on gastric glands of rabbit, which have neither lamina propria nor immune cells in the preparation.

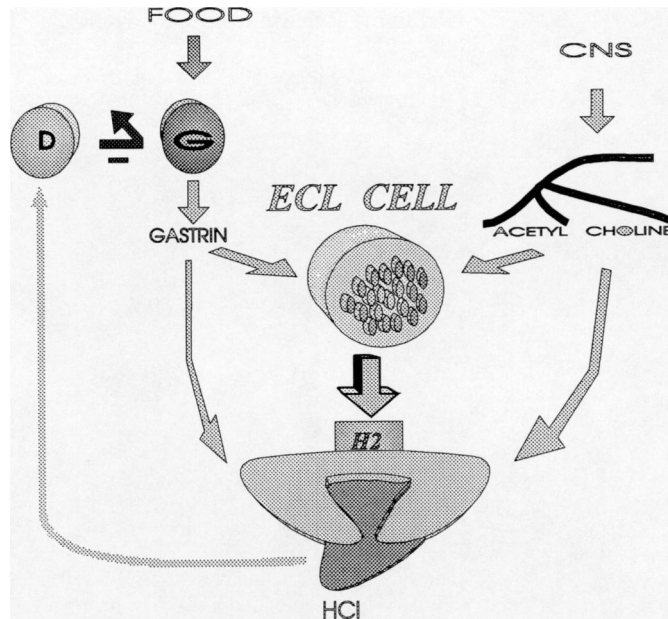


FIG. 4. A model of the role of the ECL cell in peripheral regulation of gastric acid secretion. Central nervous system stimulation results in acetylcholine release, which stimulates the ECL cell to release histamine, but acetylcholine also binds to the  $M_3$  receptor on the surface of the parietal cell. Gastrin is released by especially aromatic amino acids and stimulates the ECL cell to release histamine. Although a gastrin receptor has been found on the parietal cell, evidence indicates that this receptor is unable to stimulate acid secretion directly.

As has been discussed for the ECL cell, there are two second messenger pathways with elevation of either cAMP or  $[Ca]_i$ . Histamine binds to the  $H_2$ -receptor, a typical seven-membrane segment G protein-coupled receptor [20], with a resultant elevation of cAMP as the major second messenger. There is also an elevation of  $[Ca]_i$  that is of unknown significance in the histamine pathway [21]. Coupling to cAMP elevation is via a  $G_s$  protein, coupling to changes of  $[Ca]_i$  is presumably by a  $G_q$  or  $G_{11}$  complex, but this theory has not been experimentally established.

Acetylcholine binds to an  $M_3$  muscarinic receptor with transient elevation of  $[Ca]_i$  and then a steady-state elevation of  $[Ca]_i$  [22,23]. It appears to be the latter phase that is responsible for stimulation of acid secretion [23]. The  $M_3$  receptor is coupled to phospholipase C for activation of the transient calcium release pathway, and to an unknown pathway for calcium entry. These two pathways are determined by two states of the  $M_3$  receptor. One state, resulting in calcium entry, depends on a high-affinity acetylcholine binding site; the other is lower affinity by about one order of magnitude and appears coupled to release of calcium from intracellular stores [23]. The release pathway is activated at higher concentrations of muscarinic agonist, and it is conceivable that the high level of  $[Ca]_i$  that occurs after phospholipase C (approximately 1,000 nM) activation is inhibitory and shuts off the stimulatory effect of the steady-state elevation of  $[Ca]_i$  (approximately 200 nM).

Gastrin also has a receptor on the parietal cell, again a seven-membrane segment

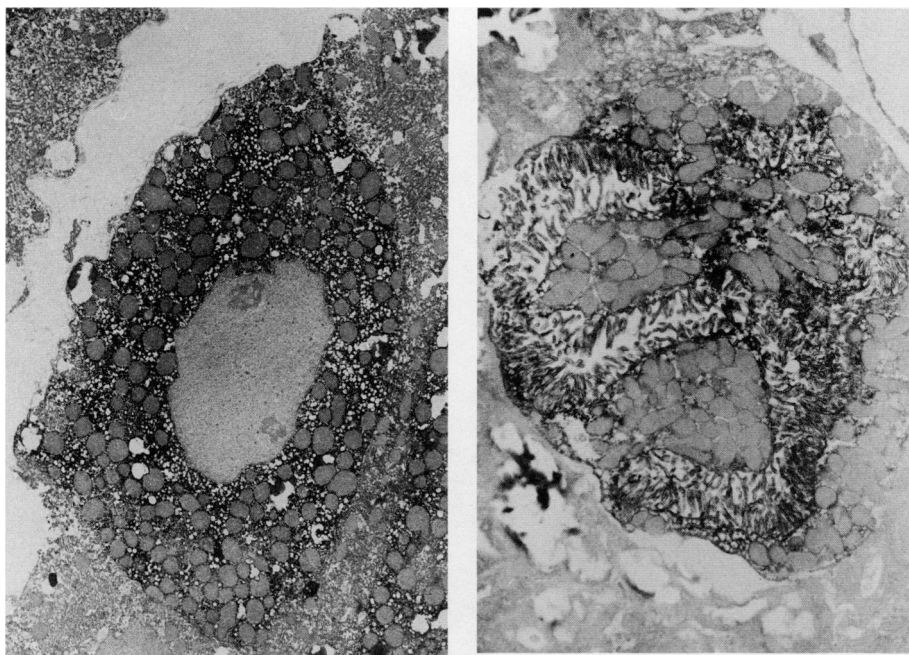


FIG. 5. An electron micrograph of a resting (*left*) and stimulated (*right*) parietal cell, stained with a monoclonal antibody against the  $\alpha$  subunit of the H,K ATPase [18]. It can be seen that the resting cell has the pump present in cytoplasmic tubulovesicles; in the stimulated cell, the pump is present in the microvilli lining the expanded secretory canaliculus.

G protein-coupled receptor, which results, as for the ECL cell, in elevation of  $[Ca]_i$  [24]; however, the role of this receptor in activation of acid secretion is controversial. On its own, it appears to be the weakest of the three in terms of stimulation. The claim has been made that gastrin activation of acid secretion requires cAMP elevation and that gastrin can potentiate either acetylcholine or histamine stimulation. The role of the gastrin receptor on the parietal cell remains controversial. A model of the receptors and their pathways is shown in Fig. 6.

The various ligands that stimulate the parietal cell converge to activate the acid pump. Along with and accompanying this activation is the morphological transformation, whereby the secretory canaliculus of the cell expands as a function of a large increase of the microvilli lining this infolding of the apical plasma membrane of the cell (see Fig. 5).

## THE ACID PUMP

### *Function of the Pump*

The acid pump of the parietal cell has been shown to be the H,K ATPase, which is a member of the phosphorylating class of ion transport ATPases. The enzyme is a heterodimer [25], with a catalytic or  $\alpha$  subunit composed of about 1,000 amino acids and a glycosylated  $\beta$  subunit of about 300 amino acids. Hydrolysis of ATP results in ion transport; the chemical reaction, hydrolysis of ATP, is scalar and is converted to vectorial ion transport by conformational changes induced in the protein. The overall transport reaction is the electroneutral exchange of cytoplasmic  $H^+$  for  $K^+$  [6]. In



## STIMULATION of PARIETAL CELL

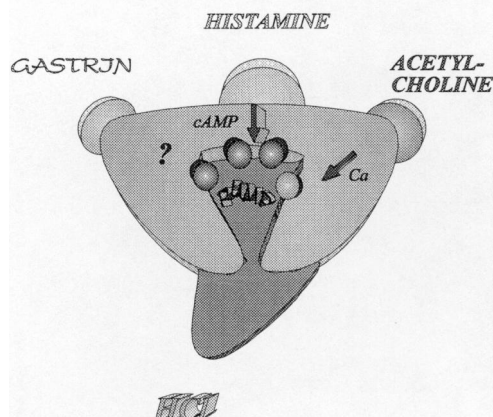


FIG. 6. A model of the receptors on the parietal cell and their second messenger pathways. Three activating receptors are illustrated. The major effect is exerted by activation of the histamine 2 receptor, which activates adenylate cyclase. Acetylcholine, by binding to the  $M_3$  receptor, results in changes in  $[Ca]_i$ . The changes in  $[Ca]_i$  due to gastrin appear not to be related directly to acid secretion.

fact, the forward  $H^+$  transporting steps and the countertransport of  $K^+$  are both electrogenic. For the pump to function, potassium must be supplied to the extracytoplasmic surface. Therefore, when associated with a potassium chloride pathway in the canalicular membrane which allows potassium chloride efflux from the parietal cell, the pump is activated, which results in secretion of hydrochloric acid at the expense of ATP breakdown.

At pH 8, the pump is able to use  $Na^+$  as a surrogate for protons, suggesting that the transported species is not the hydrogen but the hydronium ion,  $H_3O^+$  [26]. There has been considerable speculation that  $H^+$  transport across biological membranes is by means of a pathway specialized for the transport of protons, a proton wire. For example, a chain of serine OH groups in a protein would form such a proton wire.  $Na^+$  could not move along such a chain. Since  $Na^+$  is transported by the gastric ATPase, it is probably a conventional cation transport pathway that is involved, not a "proton wire." Indeed, the idea that  $H_3O^+$  is transported allows release of this cation in the same way as  $Na^+$  is released by the sodium pump without invoking specialized alterations of the  $pK_a$  of carboxylic acids in the ion binding site of the pump.

The parietal cell secretes acid by virtue of activation of the  $H^+,K^+$  ATPase. The steps involved in activation include the morphological transformation of the cell, whereby the pump moves from a cytoplasmic location in tubulovesicles to the membrane of the secretory canaliculus. The activity of the pump is determined by the access of  $K^+$  to the extracytoplasmic surface of the pump [6]. In the absence of  $K^+$  on this surface of the pump, the pump cycle stops at the level of phosphoenzyme. The potassium ion is required at this surface of the pump in order to allow dephosphorylation to occur. Hence an additional step of the activation process is activation of potassium and chlorine transporters in the pump-containing membrane (Fig. 7).

#### The Site of Acid Secretion

The morphological transformation discussed above has been illustrated in Fig. 5, where, on the left, is a resting parietal cell stained with a monoclonal antibody against the  $H,K$  ATPase  $\alpha$  subunit. It can be seen that the majority of the pump is located in the cytoplasmic tubulovesicles. On the right-hand side of Fig. 5, a

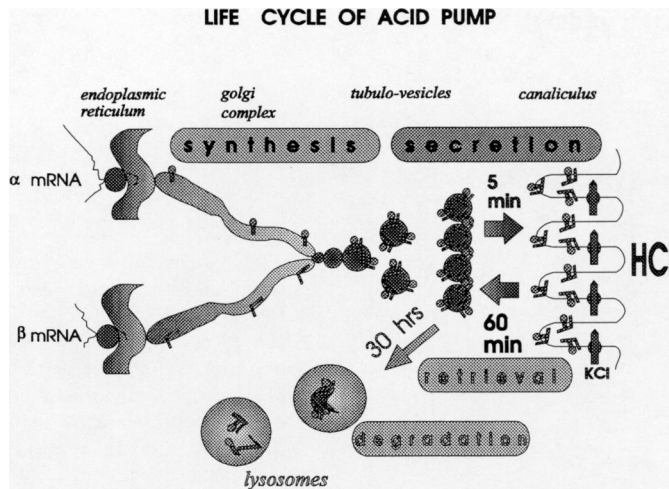


FIG. 7. A model of the biosynthesis and activation of the H,K ATPase in the parietal cell. The two subunits are synthesized on the endoplasmic reticulum and move through the Golgi complex to form the mature tubulovesicles, where the pump is present as a heterodimer. Stimulation results in formation of the microvilli of the secretory canaliculus, and the pump is now associated with the potassium chloride pathway. Loss of stimulus results in retrieval of the pump. Shown also is degradation of the pump, and the balance between synthesis and degradation gives an effective half-life of about 30 hours.

stimulated parietal cell is illustrated, stained with the same antibody. It is evident that the pump is now located in the microvilli lining the secretory canaliculus.

Acridine orange staining of the parietal cell in rabbit gastric glands has shown that the final location of acid secretion is in the secretory canaliculus, as predicted by Golgi [27]. This observation does not define the site of initiation of acid secretion. Fluorescence microscopy does not have sufficient resolution to enable detection of acidity in tubulovesicles, so this approach leaves unanswered the question as to when the association occurs between the pump and the potassium chloride pathway.

In order to detect the site of acid secretion, we take advantage of the properties of omeprazole, a clinically useful inhibitor of the H,K ATPase. Its mechanism of action is illustrated in Fig. 8. It is a weak base of  $pK_a = 4$ . Hence it will concentrate in acid spaces of less than pH 4. The only space in the body (or in rabbit gastric glands *in vitro*) that has a pH less than 4 is the acid space of the secreting parietal cell. Once in an acid environment, omeprazole is converted in an acid-catalyzed reaction to a cationic sulfenamide as illustrated in Fig. 8. This sulfenamide is a permanent cation and is therefore relatively membrane-impermeable. Sulfenamides react with protein thiols to make stable, covalent disulfides. In the case of the parietal cell and the H,K ATPase, the sulfenamide generated from omeprazole reacts exclusively with the thiols of the  $\alpha$  subunit of the H,K ATPase [28]. In fact, given its positive charge, the sulfenamide reacts only with the thiols accessible from the acid space, those available in the extracytoplasmic domain of the enzyme. Since this product is a stable covalent derivative that can form only in acid spaces, it is possible to use electron microscopic

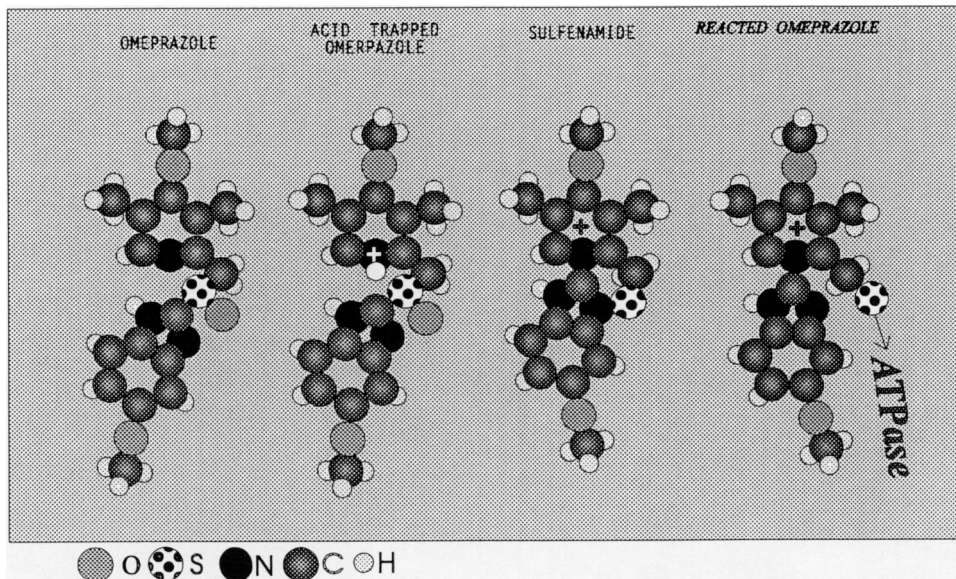


FIG. 8. The pathway of activation of omeprazole. The compound accumulates in acid due to protonation, converts to the cationic sulfenamide in an acid-catalyzed reaction, and the sulfenamide reacts with the cysteine SH groups of the catalytic subunit that are exposed to the luminal surface of the pump.

autoradiography to determine the site of stimulation of acid secretion, by determining the site of binding of omeprazole.

Rabbit gastric glands are made relatively non-secreting by treatment with the  $H_2$  antagonist, cimetidine. At zero time, omeprazole is added, along with a stimulant such as cAMP. At different times, the glands are sampled, fixed, and processed for electron microscopic autoradiography. The silver grains over the parietal cells are allocated either to a cytoplasmic, canalicular, or nuclear compartment. As illustrated in Fig. 9, increase of radioactivity over the first ten minutes is found essentially only over the canalicular compartment. If activation occurred while the pump was still in the tubulovesicular compartment, an increase of radioactivity would have been found first in this compartment, not in the canalicular compartment. This finding gives direct evidence that acid secretion starts only upon insertion of the pump into the canalicular membrane [29].

The implications as to the location of active potassium chloride transporters is that they are present only in the canalicular membrane. If activation were possible while the pump was in the cytoplasmic compartment, the cell would face the risk of cytoplasmic acidification, since with continued hydrochloric acid secretion there would be the possibility of intracellular lysis of highly acidic particles.

The  $K^+$  and  $Cl^-$  transporter(s) could be present only in the canalicular membrane, or the  $K^+$  transporter in the vesicle membrane and the  $Cl^-$  transporter in the canalicular membrane, or both in a separate vesicle population. Alternatively, active cAMP or calcium-dependent kinases might be bound to the canalicular membrane and not present in the cytoplasm. The data presented do not distinguish between these possibilities. The resting pump membrane, however, possesses neither a  $Cl^-$  nor a  $K^+$  conductance [6].

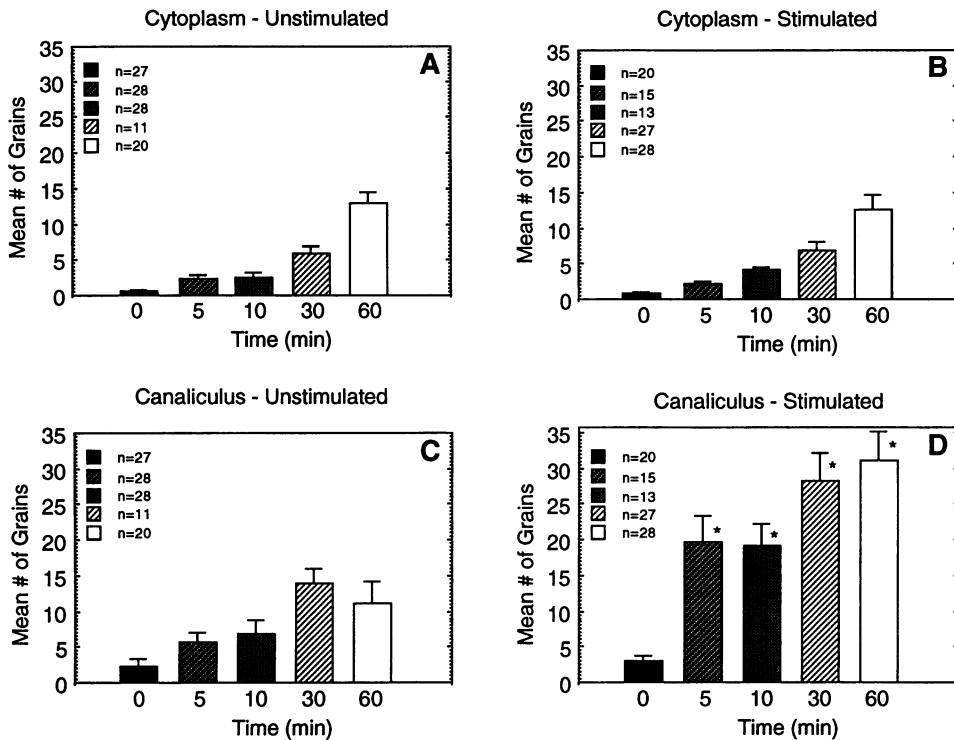


FIG. 9. The distribution of omeprazole binding in cells following stimulation of acid secretion [29]. Gastric glands were inhibited by cimetidine, and then a batch was stimulated by cAMP, and  $^3\text{H}$  omeprazole added at zero time. Samples were taken at the times indicated and the location of the omeprazole determined by electron microscopic autoradiography.

### *The Secondary Structure of the H,K ATPase*

Transport by this class of enzyme is thought to be a result of conformational changes during the cycle of phosphorylation and dephosphorylation such that the affinity and sidedness of the ion binding sites change. Thus, in the proton transport direction for the H,K ATPase, the  $E_1$  conformation binds the proton or hydronium ion from the cytoplasmic side at high affinity. With phosphorylation, the conformation changes to the  $E_2\text{P}$  form, which has low affinity for  $\text{H}^+$  and high affinity for  $\text{K}^+$  from the extracytoplasmic face of the pump, allowing release of  $\text{H}^+$  and binding of  $\text{K}^+$ . With dephosphorylation, the  $E_1\text{K}$  conformation is produced, with a low affinity for  $\text{K}^+$  with release of  $\text{K}^+$  to the cytoplasmic side, allowing rebinding of  $\text{H}^+$ . Hence pumping is the result of cycling between the  $E_1$  and  $E_2$  forms of the enzyme [30]. Figure 10 illustrates the kinetically defined steps of the pump cycle. Definition of the changes in pump structure during the conformational changes is a major goal of current research.

A description of ion transport by these pumps must come, in part, from knowledge of the membrane-spanning segments of these pumps, because ions must traverse the structure formed by the segments. Since these parts of the protein are inserted in the hydrophobic phase of the bilayer, there must be pathways allowing the passage of charged ions within the protein segments.

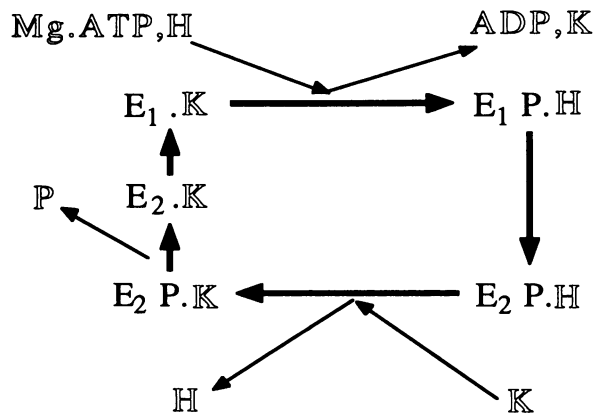


FIG. 10. The catalytic cycle of the H,K ATPase, as determined by the kinetics of phosphorylation and dephosphorylation. The pump starts its cycle with the binding site for the proton exposed to the cytosolic face, where the ion binds with high affinity. Phosphorylation then forms the E<sub>1</sub>P.H form, which converts to the E<sub>2</sub>P.H form, now with the hydrogen binding site (H<sub>3</sub>O<sup>+</sup>) exposed to the exterior. Release of the hydronium is accompanied by the binding of potassium to form the E<sub>2</sub>P.K form, which dephosphorylates to the E<sub>2</sub>K form. The E<sub>2</sub> form therefore has its ion binding sites exposed to the luminal face of the pump. The E<sub>2</sub>K form then converts to E<sub>1</sub> to restart the cycle.

The secondary structure of the EP-type ATPases has been deduced largely from hydrophathy plots. These plots have been interpreted variously. It has been suggested that the  $\alpha$  subunit has eight to ten membrane-spanning segments, whereas the  $\beta$  subunit is considered to have only a single segment. In the case of the Na,K ATPase, the first pair of hydrophobic segments long enough to be membrane-spanning in  $\alpha$  helical form, H<sub>1</sub> and H<sub>2</sub>, have been shown to be indeed membrane-spanning by virtue of the fact that this region of the enzyme determines the ouabain binding affinity of the Na,K ATPase [31], ouabain being an extracytoplasmic inhibitor of the sodium pump. The protease resistance of the 19 kDa C-terminal portion of the  $\alpha$  subunit of this enzyme has been taken to mean that it is largely membrane-embedded [32], and therefore has six membrane segments. In the case of the Ca ATPase of sarcoplasmic reticulum, it has been shown that antibodies against the region containing residues 878 to 890 react with the extracytoplasmic face of the enzyme. This region would correspond to the loop between M<sub>7</sub> and M<sub>8</sub> in the originally proposed model. The calmodulin binding domain of the Ca ATPase of plasma membrane must be cytoplasmic and is in the C-terminal region of this enzyme, which has led to a ten-membrane segment-spanning model for this enzyme [33].

In the case of the gastric H,K ATPase, various lines of evidence have been gathered to substantiate the model of Fig. 11, an eight- or ten-membrane-spanning model for the  $\alpha$  subunit.

Tryptic cleavage of intact, inside-out vesicles of the ATPase at a 1:4 enzyme-to-protein ratio, followed by SDS solubilization of the residual membranes, theoretically should yield the membrane-spanning pairs. Labeling the SH groups present in this fraction with fluorescein maleimide allows visualization of the segments present.

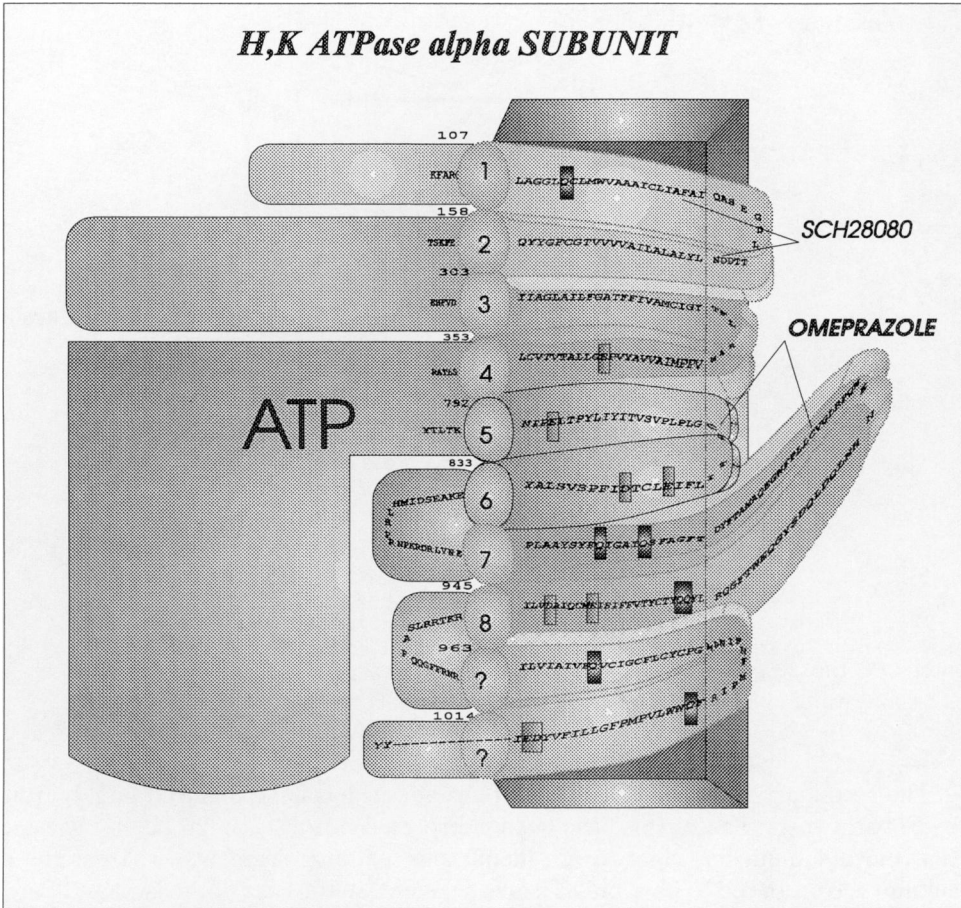


FIG. 11. A model of the secondary structure of the  $\alpha$  subunit of the pump, illustrating eight and perhaps ten membrane-spanning segments, along with the site of binding of the potassium competitive inhibitor SCH28080 and the sites of binding of omeprazole.

As illustrated in Fig. 12, fluorescent segment pairs were found corresponding to  $H_1$  through  $H_9$ . The  $H_9$  and  $H_{10}$  sequences were, however, absent after digestion and washing, indicating that perhaps these sequences were not membrane-spanning or that the cysteines were not available for labeling by fluorescein maleimide [28].

A molecular biological approach has provided evidence that the  $H_9$  and  $H_{10}$  segments are able to traverse the lipid bilayer. Here a construct was made of mRNA where the N-terminal part of the  $\alpha$  subunit was interrupted by an insert containing either  $H_9$  or  $H_9$  and  $H_{10}$  message, followed by the C-terminal part of the  $\beta$  subunit, which contains seven potential glycosylation sites. If  $H_9$  was present in an *in vitro* translation system with dog microsomes, glycosylation of the product was observed, showing that  $H_9$  crosses the microsomal membrane, allowing glycosylation of the  $\beta$  subunit to occur. Thus  $H_9$  can act as a signal transfer sequence. When  $H_9$  and  $H_{10}$  are inserted into the mRNA construct, no glycosylation is observed, showing that  $H_{10}$  can act as a stop transfer sequence, characteristic of the second of a pair of membrane-spanning segments [34]. The model illustrated therefore shows the presence of an

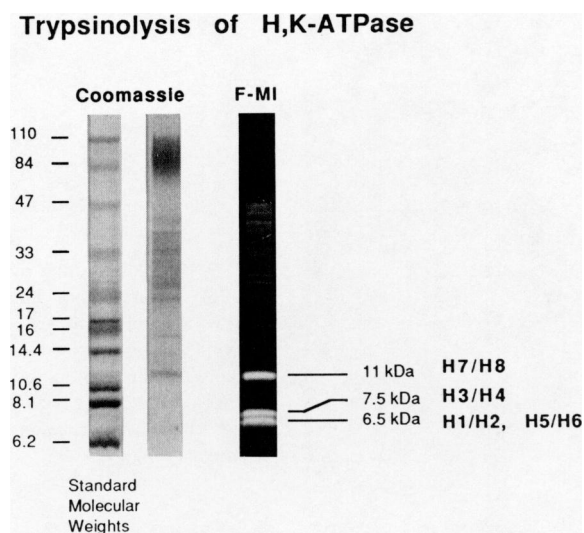


FIG. 12. The membrane segments of the pump as determined by tryptic hydrolysis, separation of the membrane-retained segments, labeling these with fluorescein maleimide after solubilization, and running on SDS gels [28,37]. The labeled bands were sequenced and represent the eight first membrane-spanning segments of the catalytic subunit.

additional pair of segments, not found by the biochemical methods we have employed [28].

Hence these approaches provide direct evidence for an eight- and perhaps a ten-membrane-spanning model. It may be noted that these segments correspond closely to the segments proposed for the Ca ATPase, but do not correspond to the eight segments originally suggested by analysis of hydropathy of the H,K ATPase [7].

#### *Inhibitor Sites on the H,K ATPase*

Since this ATPase is responsible for acid secretion and is, indeed, the “final common pathway” and acid is a sine qua non for upper gastrointestinal ulcer diseases, various types of compounds have been and are being developed for treatment of these diseases. These compounds may be classified into  $K^+$  competitive and covalent types.

**$K^+$  Competitive** A typical  $K^+$  competitive ATPase inhibitor, the aryl quinoline, MDPQ, is illustrated in Fig. 13. It is a N-containing heterocyclic compound like the imidazopyridine, SCH28080. The effect of this class of compound is exerted on the extracytoplasmic face of the pump [35]. Using a photoaffinity analog of SCH28080, MeDAZIP<sup>+</sup>, it has been shown that binding occurs at the extracytoplasmic loop between  $M_1$  and  $M_2$ , the same region that binds ouabain in the Na,K ATPase [36]. Molecular modeling studies argue that these compounds dock at phe<sup>124</sup> and asp<sup>136</sup>. This finding also provides direct evidence for the extracytoplasmic location of the binding site between  $M_1$  and  $M_2$ .

Using MDPQ as a fluorescent probe of conformation, it was found that fluorescence was increased upon the addition of ATP in the presence of magnesium, due to the formation of an  $E_2P \cdot [I]$  conformation of the enzyme [37]. This finding can be interpreted as due to an increase in the hydrophobicity of the environment of the  $K^+$  competitive inhibitor. The binding site is calculated to be at the interface between the phospholipid head groups and the fatty acids of the bilayer, which implies that part of the conformational change involved in transport is tilting of the helices forming  $M_1$  and  $M_2$ .

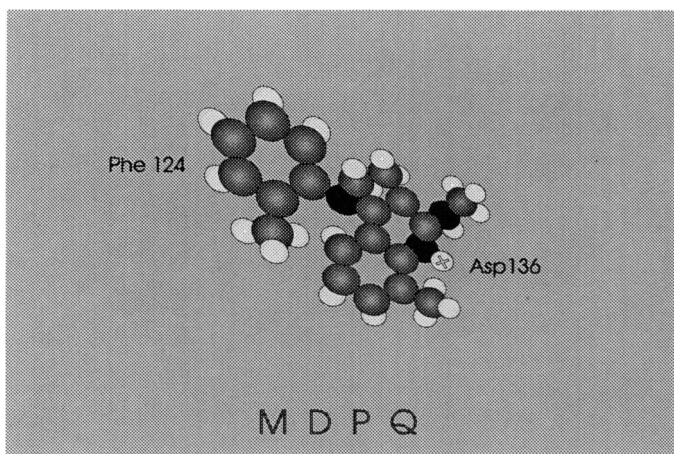


FIG. 13. The structure of MDPQ, a potassium competitive aryl quinoline [36], showing its position when docked, as the protonated species to phe<sup>124</sup> in M<sub>1</sub> and asp<sup>136</sup> in the loop between M<sub>1</sub> and M<sub>2</sub>.

Another fluorescent probe, fluorescein isothiocyanite (FITC), has been shown to bind to the cytoplasmic domain at lys<sup>516</sup>. When Na<sup>+</sup> is added, the environment of this probe becomes more hydrophobic as a function of formation of the E<sub>1</sub>·Na conformation. The addition of K<sup>+</sup> decreases fluorescence as a function of the E<sub>2</sub>·K conformation. The changes can be interpreted as a reciprocal conformational change of the cytoplasmic domain and the extracytoplasmic domain between M<sub>1</sub> and M<sub>2</sub> during the transport cycle.

Hence these fluorescent data suggest membraneward movement of the large intracellular loop of the protein between H<sub>4</sub> and H<sub>5</sub> in the E<sub>1</sub> direction and membraneward movement of the extracytoplasmic region between M<sub>1</sub> and M<sub>2</sub> in the E<sub>2</sub> direction. Other parts of the extracytoplasmic face can also be involved as indicated by the effects of binding of the covalent class of pump inhibitor discussed below.

*Covalent Inhibitors* There are a variety of these inhibitors on the market or under development for treatment of ulcer disease. Omeprazole was the first of these compounds developed for clinical use, and lansoprazole and pantoprazole are very similar in structure [38]. Their general mechanism has been illustrated above. From this information, it can be deduced that these compounds react on the extracellular face of the enzyme with available cysteines.

Tryptic hydrolysis and mapping of the cysteines reacting with omeprazole showed that cysteine 813 and/or 822 react as well as cysteine 892, whereas an analog, pantoprazole, reacts only with cysteine 813 and/or cysteine 822, to give the same inhibition [28,38]. The cysteines 813 and 822 are predicted to be in or between M<sub>5</sub> and M<sub>6</sub> and the cysteine 892 in the large extracytoplasmic loop between M<sub>7</sub> and M<sub>8</sub>, as illustrated in Fig. 11.

The binding of the cationic sulfenamide derived from omeprazole blocks phosphorylation and ATPase/pNPPase activity, as if the enzyme were locked into an E<sub>2</sub>·[I] configuration. This finding would argue that motion of the M<sub>5</sub>/M<sub>6</sub> region is also part of the conformational changes involved in transport.

Depending on the compound used, it appears that one or both of the cysteines at 813 and 822 react. It is probably these, rather than the cysteine 892, thought to be distant from membrane interface, that determine the inhibitory effect of omeprazole



TABLE 1  
H<sub>2</sub>-Receptor Antagonists and Acid Pump Inhibitors

| Similarities                               | Differences  |
|--|--|
| Reversible inhibition of acid secretion    | Molecular target   |
| No side effects                            | Efficacy and duration of action                                  |
| Few adverse reactions                      | Effective in GERD,NSAID  |
| Hypergastrinemia                           | Effective with single antibiotic in <i>H. pylori</i> eradication |
| Effective in duodenal ulcer, gastric ulcer |  |

and its analogs. For example, pantoprazole reacts only with the cysteines in the M<sub>5</sub>/M<sub>6</sub> region, but has an identical inhibitory action on the H,K ATPase [38]. The binding of pantoprazole alters the tryptic cleavage pattern of the enzyme, whereby the major site of tryptic cleavage at the N-terminal end of the M<sub>5</sub> segment moves from position 776 to position 792. Hence the binding of pantoprazole to cysteine 822 alters the conformation of this segment of the protein, another example of a conformational change in a membrane-spanning segment of the pump.

#### *Clinical Implications*

H<sub>2</sub>-receptor antagonists have been in use for many years, with excellent results in the treatment of duodenal ulcer, good results in gastric ulcer, and good results in mild reflux disease. It would seem, however, that the multiplicity of pathways available to the parietal cell for acid stimulation prevent these compounds from reliably reducing acid secretion to levels optimal for the healing of acid-induced damage in gastric ulcer, severe reflux disease, non-steroidal anti-inflammatory drugs (NSAID)-induced ulcers and, of course, in Zollinger-Ellison syndrome. In contrast, the acid pump inhibitor class of drug is insensitive to the nature of the stimulus and with covalent reaction gives long-lasting effective inhibition of acid secretion. These drugs are therefore able to optimize the pH of either lumen or gastric wall so as to achieve the most rapid healing available with a single modality of therapy. It is interesting to compare the properties of the two classes of compound, as given in Table 1, where similarities and differences are shown. Both sets of drugs have not only derived from a better understanding of acid secretion, but have in their turn contributed to understanding of gastric physiology and biochemistry.

#### *Side Effects of Downregulation of Acid Secretion*

The introduction of H<sub>2</sub>-receptor antagonists, while revolutionizing medical therapy of ulcer disease, led initially to concerns about the effects of acid suppression. For example, bacterial overgrowth of the stomach was thought to be a possible result of H<sub>2</sub>-receptor antagonist treatment, bacterial overgrowth then leading to nitrosamine production. Nitrosamines are carcinogens. These fears turned out to be unjustified, and the H<sub>2</sub> drugs are safe and effective compounds.

The introduction of omeprazole as the first acid pump antagonist led to further fears that the compound was a carcinogen. This apprehension was based on the finding that, in long-term, pre-clinical studies in rats, after two years' treatment, ECL carcinoids (i.e., nests of ECL cells) were found in the gastric mucosa. A variety of studies have shown that elevation of gastrin is a necessary condition for the development of these carcinoids, and that it is the hypergastrinemia induced by acid

### EXPERIMENTAL PROOF of GASTRIN THEORY

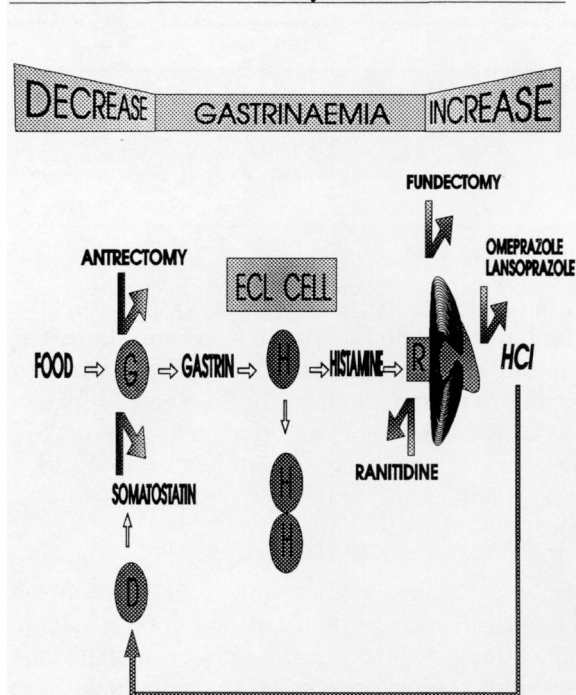


FIG. 14. A figure summarizing the various experiments performed to show that the carcinoid induction in rats by omeprazole is due to the severe hypergastrinemia induced in this model, and not to direct action of omeprazole [38]. Hence any maneuvers which reduce acid secretion, benzimidazoles, ranitidine, fundectomy, increase gastrin and ECL-cell growth; maneuvers such as antrectomy, which reduce gastrin levels, inhibit these effects.

suppression in the presence of food that results in the carcinoids in the rat. For example, high-dose ranitidine produced the same number of carcinoids as did omeprazole [39]. An illustration summarizing the various studies that influenced ECL-cell growth is presented in Fig. 14. More recently, it has been possible to show that gastrin administration to rat ECL cells stimulated cell growth as measured by incorporation of the thymidine analog, bromo-deoxyuridine [15]. These data substantiate the gastrin mechanism for induction of ECL-cell hyperplasia *in vivo*.

Long-term surveillance of a group of about 140 patients treated with high-dose omeprazole for seven years has shown omeprazole not to have a likelihood in man for the development of carcinoids. As for the H<sub>2</sub> antagonists, adverse events with this drug are also rare.

### THE FUTURE

The medical control of acid secretion has advanced considerably in this century. In terms of receptor antagonists, the relatively ineffective, nonspecific muscarinic antagonist, atropine, has been replaced by the selective, more effective, H<sub>2</sub> antagonists. In turn, these substances are being replaced by the selective, more efficacious, acid pump inhibitors such as omeprazole or lansoprazole. Further development of the K<sup>+</sup> competitive inhibitor is continuing, but perhaps acid secretory control has reached its zenith, and focus will shift to improved healing of ulcer disease and prevention of disease by eradication of *H. pylori*.

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