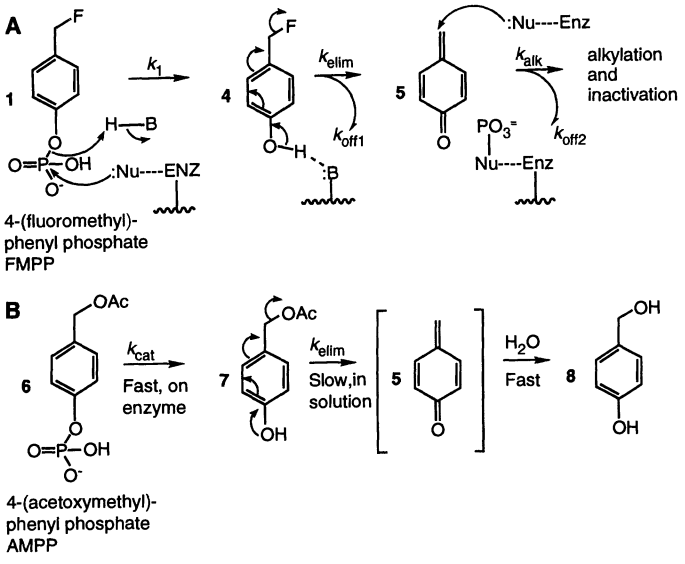

Fig. 3. Proposed mechanism of FMPP and AMPP hydrolysis and PAP inactivation. (**A**) Proposed mechanism of PAP inactivation by FMPP. Hydrolysis of phosphate esters by PAP is thought to proceed by attack on the phosphoros by an active site nucleophile (:Nu-ENZ). The leaving group alcohol is presumably protonated by an active site acid (H–B). Elimination of HX from the intermediate (**4**) may be accelerated by deprotonation of the phenol by the conjugate base of the active site acid (:B). Attack on the quinone methide by a second active site nucleophile leads to inactivation of the enzyme. (**B**) Mechanism of AMPP hydrolysis. AMPP is hydrolysed by PAP, but because the elimination of acetate is slower than that of fluoride, the intermediate (**7**) is released into solution where the resulting quinone methide is trapped by solvent.

by functioning as an affinity reagent, we synthesized and assayed phosphonate (**2**) (Fig. 1). Although this phosphonate does not contain a scissile P–O bond and therefore cannot undergo enzyme-catalyzed hydrolysis, it bears a strong structural resemblance to FMPP and is a competitive inhibitor of PAP. Incubation of **2** with PAP resulted in virtually no time-dependent enzyme inactivation, even after 20 min (Fig. 2B). This experiment suggests that PAP inactivation by FMPP requires cleavage of a P–O bond and that the benzylic fluoride moiety of FMPP is not a sufficiently reactive electrophile to bring about alkylation of the enzyme. It appears that FMPP selectively inactivates phosphatases with a particular affinity for aryl phosphates; it does not, for example, inactivate alkaline phosphatase, which displays no specificity for aryl phosphates, even though FMPP is a substrate for the enzyme (*13*).

In principle, the inactivation motif exemplified by FMPP could be used to design very specific PTPase inactivators. This motif could be incorporated into a phosphotyrosine residue (**3**) (Fig. 1) with minimum structural perturbation of the amino acid by the simple replacement of one (or both) of the benzylic hydrogens of the phosphotyrosine moiety with fluorine. The modified tyrosine phosphate might then be incorporated into a peptide sequence that is a known substrate of a PTPase. Inhibitors such as these should be most selective for phosphatases that preferentially hydrolyze the specific peptide. Finally, our results demonstrate the feasibility of designing potent inactivators of phosphatases, even when no structural information about the enzyme is available.

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15. Inorganic anions such as azide show complex inhibitory behavior toward PAP. Azide slightly slows PAP inactivation but also slows the normal turnover rate of the enzyme. Azide-mediated inhibition of the inactivation of PAP by FMPP shows saturation kinetics, which implies that the inhibition is a function of the interaction of PAP and azide rather than the preferential trapping of an exogenous electrophile by azide.

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24 May 1993; accepted 4 October 1993

TECHNICAL COMMENTS

Actions of Antiulcer Drugs

Éva Mezey and Miklós Palkovits (*1*) state that acid secretion stimuli act indirectly on epithelial cells. Using in situ hybridization techniques, they detected messenger RNAs for histamine, gastrin, and muscarinic receptors only in immunocytes of the lamina propria. We take issue with this interpretation of their data. We and others have found that parietal cells have receptors for at least acetylcholine and histamine. Mezey and Palkovits state that preparations of isolated parietal cells are sufficiently contaminated with immunocytes so that secondary stimulation by mediators released from the immunocytes could occur. However, when viewing isolated parietal cells from the gastric gland in a perfusion chamber with an imaging microscope, one sees that they respond tothe addition of carbachol with changes in the concentration of Ca ions $[Ca]_i$ (*2*). With this experimental protocol, it is difficult to conclude other than that carbachol acts directly on parietal cells. Contamination with adhering immunocytes would be apparent; our preparation is free of lamina propria, the stated location of the immunocytes of interest. An intermediate messenger would be diluted and removed by the perfusion.

Several of us have shown pharmacologically (*2*), in studies of binding and of cellular response, that isolated parietal cells have no functional muscarinic receptors other than that for M3. It has also been shown (*3*) with the use of polymerase chain reaction that gastric glands [a mixture of peptic, parietal cells and a few enterochromaffin-like (ECL) cells] express only a receptor for the m3 muscarinic subtype, and not those for m1, m2, m4, or m5 subtypes. Purified (about 90% pure) parietal cells also show the presence of only m3 receptors (*3*). The m1 subtype is absent in figure 2B of the study by Mezey and Palkovits (*1*), indicating that immunocytes did not contaminate gastric gland or parietal cell preparations.

More recently, several of us have shown that a suspension of purified (70 to 90% pure) ECL cells (considered to be the source of the histamine necessary for most of the gastrin response and some of the cholinergic response of acid secretion) releases histamine when

gastrin or carbachol is added (*4*). When this cell-type is observed in a perfusion chamber, it is seen to respond to gastrin by an elevation of $[Ca]_i$, again showing that it has a gastrin receptor (*4*) (perfusion would remove any intermediate transmitter). It may be that at least some of the in situ hybridization results seen by Mezey and Palkovits (*1*) results from the presence of ECL cells rather than immunocytes. The figures in their study do not exclude this possibility.

We conclude that parietal cells have muscarinic receptors and that ECL cells have gastrin receptors. A key problem with this report (*1*) is that it does not show evidence of messenger RNA for receptors in cells known to express those receptors.

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17 December 1992; accepted 9 June 1993

The regulation of gastric acid secretion has been investigated extensively. Acetylcholine, gastrin, and histamine are the most important regulatory factors (*1*), although the relative importance of these agents is not clear. Histamine is synthesized by a specific enzyme, L-histidine deacarboxylase (HDC). Immunocytochemical evidence strongly suggests that both histamine (*2*, *3*) and HDC (*4*) are located in ECL cells in the basal parts of gastric glands. HDC was recently cloned (*5*), and oligonucleotide probes were used to detect its messenger RNA in rat brain in tuberomammillary neurons (*6*, *7*), which are known to contain histamine (*8*). When we applied these highly specific oligonucleotide probes to sections of rat stomach, we found that both HDC probes labeled not only the basal parts of the gastric glands but also several macrophage-like cells in the lamina propria under the mucosa and a few similar cells in the area of gastric glands (Fig. 1A). Hybridization was carried out (*6*); as a control probe, we used *Staphylococcus aureus* chloramphenicol acetyltransferase probe, which is of similar length. This probe yielded a distribution of silver grains in the lamina propria similar to that produced by the specific HDC probes, but labeling in the basal parts of gastric glands did not occur (Fig. 1B).

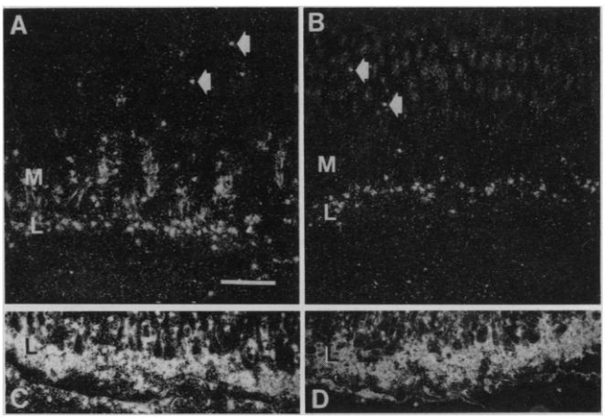

Fig. 1. Dark-field micrographs show the distribution of silver grains after in situ hybridization on the rat stomach with different oligonucleotides: M, mucosa; L, lamina propria. Scale bar = 100μm. (**A**) Hybridization with an oligonucleotide complementary to nucleotides 583 to 632 of the rat histidine decarboxylase. The mucosal grains correspond to the location of basal parts of gastric glands. In addition, grains are seen in the lamina propria. Some nonspecifically labeled cells in the mucosa are labeled with arrows. (**B**) Hybridization with a 50-nucleotide oligomer complementary to *S. aureus* chloramphenicol acetyltransferase. There are no grains in the mucosa, but the lamina propria contains grains similar to those in (A). Arrows indicate some nonspecifically labeled cells. (**C**) Hybridization with a 50-mer oligonucleotide complementary to nucleotides 13 to 62 of the human H2 receptor. A large number of silver grains are seen in the lamina propria. In the mucosa, the density of reactive cells is lower. (**D**) A 50-mer oligonucleotide complementary to the same part of the human H2 receptor in sense orientation. The distribution of the grains is similar to that in (C). (A) and (B) are from adjacent sections, as are (C) and (D).

Only single histamine- or HDC-immunoreactive cells have been shown to be present in the lamina propria of the rat stomach (*2*, *9*). In an attempt to localize H2 receptors in the rat stomach, we prepared 50-nucleotide oligomers that were complementary to different regions of the human H2 receptor (*10*). The same probes in sense orientation were used as control probes. The probes were labeled with terminal transferase (6). There was essentially no difference in the distribution of silver grains in sections hybridized with probes in antisense or sense orientation (Fig. 1, C and D). Silver grains had accumulated thickly in the lamina propria, but they were also scattered throughout the mucosa. These results suggest that cells in the lamina propria of the rat stomach bind some ^{35}S-labeled oligonucleotides nonspecifically.

Mezey and Palkovits describe (*11*) the presence of histamine H2, muscarinic acetylcholine M1 to M5, gastrin, and dopamine D1 to D5 receptors in macrophages in the lamina propria of rat stomach. They conclude that current models describing the regulation of gastric acid secretion may need revision. However, their report did not show control sections hybridized with control probes labeled to the same specific activity, the presence of receptor proteins in the macrophages, or a physiological effect mediated through the immune cells. We suggest that hybridization results obtained with oligonucleotide probes in the lamina propria of the stomach and intestine be interpreted with care because nonspecific labeling can occur.

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3 May 1993; accepted 3 September 1993

Response: We would like to thank our colleagues for their valuable comments about our report (*1*). Scott *et al.* conclude from their data that "parietal cells have muscarinic receptors and that ECL cells have gastrin receptors." They state that a key problem with our report was that it did not show "evidence of messenger RNA for receptors in cells known

to express those receptors." They also say that parietal cells have M3 acetylcholine receptors, having observed that isolated gastric gland parietal cells respond to the addition of carbachol by increasing their influx of Ca. We have observed some immunocytes in close proximity to epithelial cells—especially after immobilization stress—so we would expect them to be present in isolated gastric glands. Their presence or absence might be responsible for the great variation between the responses of individual parietal cells that Scott *et al.* have observed. An intermediate messenger released by the stimulation of immunocytes could indeed be diluted and removed by perfusion, but a messenger released into the narrow cleft separating the immune and epithelial cells might be refractory to perfusion. To show definitely that epithelial cells have acetylcholine (and histamine) receptors, one would have to study dissociated cells.

This is not to say that convincing evidence for the presence of acetylcholine receptors on parietal cells, or gastrin receptors on enterochromaffin-like cells (another population of epithelial cells), would be totally unexpected. In fact, our latest studies (*2*), which use more sensitive RNA probes, suggest that in the rat all (and not only parietal) gastric epithelial cells may express the M3 receptor mRNA, in addition to the immunocytes described in our report. However, we were unable to detect mRNA for most of the other receptors we studied (including H2) in epithelial cells (possibly because of the low concentration of mRNA (*1*). We could easily detect these receptors in immune cells of the gastric and duodenal lamina propria of humans and rats. The interaction between the immune and epithelial cells of the gastrointestinal system should be examined in more detail and the effect of agonists and antagonists on both populations of cells studied.

We have performed further control studies to validate the in situ hybridization technique that we used in our report. Although the intensity of the signal varied, the mRNAs we described (*1*) were still present in immunocytes of the gastric lamina propria. Even if the parietal cells have neurotransmitter receptors, our data would still suggest that immune cells in the stomach (and duodenum) are targeted by drugs that act on these receptors.

We thank Panula and Wasowicz for their thoughtful criticism of our methods (*1*). They observed a positive hybridization reaction in the gastric lamina propria when they used an oligonucleotide probe complementary to histidine decarboxylase mRNA and also when they used a control probe. Furthermore, they found the same hybridization signal in the lamina propria of the stomach when they used sense or antisense oligonucleotide probes that were directed against the human H2 receptor sequence labeled with ^{35}S. Because they used a human probe to study the rat, it is difficult to interpret their results. We have seen that lamina propria cells can bind probes nonspecifically, and to avoid this problem we have performed the following controls.

We used sense strand probes labeled to the same specific activity as the antisense ones for the D4 and D5 receptors and have not seen any labeling in the lamina propria (as we mentioned in our report, but did not demonstrate because of space constraints). Although our sequences were based on the human complementary DNA (cDNA), they had been previously tried and successfully used to study rat tissues.

Since our report was published, we have used complementary RNA (cRNA) probes to investigate human H2 (in human tissue), rat M1 and M3, rat D3 and D5, and rat gastrin receptors. We have found lamina propria cells that tested positive for H2, M1, M3, D4, and D5 and have visualized epithelial cells with the M1, M3, and D5, but not the H2, receptor probe. The M3 probe hybridized to most if not all epithelial cells; the D4 bound to basal cells of the gastric glands; the M1 probe gave a weak signal in the part of epithelium where parietal cells are located (*2*).

We used several ^{35}S-labeled probes that did not label lamina propria cells [including probes for the TRH receptor, somatostatin (peptide), serotonin 1A receptor, dopamine-β-hydroxylase, and phenylethanolamine-N-methyl transferase mRNAs]. The somatostatin probe labeled only scattered endothelial cells, and a probe complementary to the cDNA of the proton pump (the site of action of omeprazole in producing its antiulcer effects) labeled only the parietal cell layer (Fig. 1).

Whenever it was possible to do so, we confirmed our results with immunostaining and have done so with TH, substance P, neurotensin, and calcitonin-gene related peptide (CGRP). We have not found any discrepancies between the data obtained with in situ hybridization histochemistry and that obtained with immunocytochemistry. Because there are no commercially available antibodies to the receptors that we studied, we could not confirm our receptor in situ results with immunostaining.

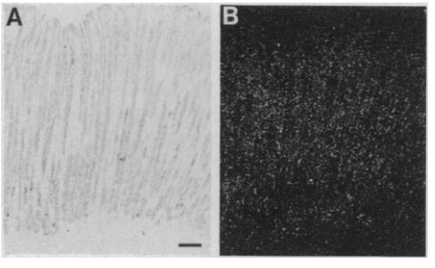

Fig. 1. In situ hybridization histochemistry with a ^{35}S-labeled oligonucleotide probe used to detect the proton pump mRNA (*4*). A longitudinal section of the rat stomach shows a positive autoradiographic signal in the parietal cell layer of the epithelium. (**A**) bright-field, (**B**) dark-field illumination. Bar, 100 μm.

Panula and Wasowicz mention correctly that our "report did not show . . . a physiological effect mediated through the immune cells." However, we did state (*1*) that, in stressed animals that developed ulcers, we observed a migration of immune cells from the base of the gastric gland toward the lumen. Many of these immunocytes seemed to attach to the epithelial cells. On the basis of this observation, we suggested that the epithelial and lamina propria cells might be interacting.

We have recently established a likely reason for the unusually intense labeling of some lamina propria cells: Briefly, an amplification of the radioactive (^{35}S) signal in phagocytic cells results from high amounts of oxidative enzymes being present. There seems to be a *specific* binding of the probe to the mRNA, then signal amplification. Infrequently, there is a partial match between the probe and a nontargeted mRNA species, and this nonspecific binding is amplified in phagocytic cells. As a result of the amplification, the nonspecific signal—which would normally not be seen—becomes detectable (*3*). We have repeated some of our experiments using ^{33}P instead of ^{35}S to avoid this amplification. Thus far, the results have been qualitatively similar, but the signals have been weaker than those seen with ^{35}S labeling.

Although we maintain that the lamina propria cells in the stomach bear the receptors that we studied, we agree with Panula and Wasowitz that one must be cautious when trying to detect radioactive signals in cells of the lamina propria.

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