surface-stainable VSV-G (IND) after incubation with cells or to prime mice for cytotoxic T cells or efficient T cell help (*27*). Peritoneal macrophages were incubated with UV- or formaldehyde-inactivated VSV-IND 90 min at 37°C at a multiplicity of 1. The supernatant containing unadsorbed virus was saved, and the cells were washed three times. Both cells and supernatant were injected directly into the spleen of ICR mice to avoid loss of cells in the lung.

21. Suppressor T cells are not involved because there are no CD8+ T cells that are glycoprotein-specific in *H-2*^{b} mice [J. W. Yewdell *et al.*, *J. Exp. Med.* **163**, 1529 (1986); T. M. Kündig *et al.*, *J. Virol.* **67**, 3680 (1993)]. Also, B cells able to be activated must be present in KINDG mice because they respond promptly to VSV-IND virus [which may be even more apt at inducing CD8+ T cells than soluble VSV-G (IND), but obviously this suppression does not happen here].
22. The concentration of glycoprotein needed to compete for 50% of antibody binding in the glycoprotein-specific ELISA was determined as described [S. Rath, C. M. Stanley, M. W. Steward, *J. Immunol. Methods* **106**, 245 (1988)]. In brief, graded amounts of purified viral particles were incubated with diluted sera of VSV-IND–immunized mice. Serum dilutions leading to less than the half-maximal absorbance without competing glycoprotein were used in order to avoid antibody excess.
23. Mice were immunized with 2 × 10^{6} PFU of VSV-IND, and spleens were removed 8 days later. Spleen cell suspensions were prepared, and 4 × 10^{5} cells were incubated in 96-well plates in the presence of various amounts of purified VSV-G (IND) in serum-free Ventrex medium (NBS Scientific Biologicals, Portland, ME) at 37°C. Three days later, the cells were pulsed for 8 hours with [^{3}H]thymidine (1 mCi per well).
24. R. M. Zinkernagel *et al.*, *Immunol. Rev.* **122**, 133 (1991).
25. B. Odermatt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8252 (1991).
26. R. R. Wagner, *The Rhabdoviruses* (Plenum, New York, 1987).
27. M. F. Bachmann *et al.*, *J. Virol.* **67**, 3917 (1993).
28. This experiment shows that neutralizing antibodies induced by VSV-IND particles in KINDG mice also react with the poorly organized form of VSV-G (IND) and thus do not represent new antibodies against determinants that are unique to the virion and therefore would be anti-foreign. This notion is supported by the fact that the neutralizing activity of neutralizing polyclonal and a collection of about 60 monoclonal VSV-IND antibodies can readily be absorbed by infected cells or purified G (IND). In addition, it appears that there is only one single neutralizing epitope on VSV [H. P. Roost, thesis, University of Zürich (1991)].
29. Thus, optimal B cell induction is achieved with repetitive antigens, and induction of tolerance with repetitive antigens, for example, flagellin [G. Möller, *Immunol. Rev.* **23**, 126 (1975)], may either reflect interaction with, and deletion of, immature B cells or exhaustive induction of mature B cells, particularly in the absence of T help.
30. J. Lindstrom, *Annu. Rev. Immunol.* **3**, 109 (1985).
31. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *Science* **182**, 293 (1973).
32. R. Z. Dintzis *et al.*, *J. Immunol.* **143**, 1239 (1989); H. M. Dintzis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3671 (1976).
33. S. Charan and R. M. Zinkernagel, *J. Immunol.* **136**, 3057 (1986); plates were coated with purified VSV-G (IND).
34. The serum neutralization test was performed as described [H. P. Roost, S. Charan, R. M. Zinkernagel, *Eur. J. Immunol.* **20**, 2547 (1990)]. To determine IgG titers, we pretreated undiluted serum with an equal volume of 0.1 M 2-mercaptoethanol in saline. Unreduced samples were taken as IgM titers only if the corresponding reduced samples had a titer at least one-quarter its size—that is, when the IgG present in the unreduced sample could be neglected.
35. Mice were kept and experiments were performed according to cantonal and federal law in Switzerland.
36. For the CD4 depletion of mice, on days 3 and 1 before immunization the mice were injected with two doses of 1 mg of YTS 191.1. The depleted CD4+ T cell population was determined to be below the detection level by fluorescence-activated cell sorter analysis. Functional depletion was confirmed in control mice by complete abrogation of the IgM to IgG switch of neutralizing antibodies against VSV (*18*).
37. VSV-N–specific IgG ELISA titers were determined in the serum (*33*).
38. Mice were immunized with 2 × 10^{6} PFU of VSV-IND. Twenty-one days later, blood was taken and spleen and bone marrow cells were isolated. VSV-G (IND)–specific ELISA titers were determined in the serum as described (*33*). VSV-G (IND)–specific AFCs were enumerated in spleen and bone marrow as described [D. Moskophidis and F. Lehmann-Grube, *J. Immunol.* **133**, 3366 (1984); plates (Greiner, Frickenhausen, Germany) were coated with purified VSV-G (IND)]. To calculate the reciprocal of the ELISA titer per the number of AFCs, we look for groups of three mice the sum of AFCs from spleen and bone marrow per 10^{6} lymphocytes. A similar method has been used by C. C. Goodnow and co-workers (*2*). Because in the VSV system AFC tests cannot be performed for the neutralizing determinant only, we determined AFCs and ELISA titers specific to the whole VSV-G (IND) molecule. We have found that if purified VSV-IND is used to coat plates for ELIspot assays to assess AFCs, more than 50% of VSV-specific AFCs are specific for the neutralizing determinant of VSV-G (M. F. Bachmann, unpublished results). Neutralization assays and ELISA tests against VSV-G therefore appear to measure the same antibodies and in fact give comparable results in the VSV system. To determine the glycoprotein concentration competing for 50% of antibody binding in the ELISA (*22*), we analyzed the diluted sera of three mice per group 21 days after immunization with VSV-IND.
39. The baculovirus expressing the G (IND) of VSV was a generous gift of D. H. Bishop, Institute of Virology and Environmental Microbiology, Oxford, UK. Recombinant vaccinia viruses expressing the G and N of VSV were generous gifts of B. Moss, Laboratory of Viral Diseases, NIH, Bethesda, MD. The hybridoma cell line YTS 191.1 was a generous gift of H. Waldmann, Cambridge, UK. Supported by grants from the Swiss National Science Foundation (31-32179.91) and the Kanton Zürich. We thank A. Althage and H. Pircher for critically reading the manuscript and Y. Deflorin for excellent secretarial assistance.

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Mechanism-Based Inactivation of Prostatic Acid Phosphatase

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Protein phosphatases play important roles in the regulation of cell growth and metabolism, yet little is known about their enzymatic mechanism. By extrapolation from data on inhibitors of other types of hydrolases, an inhibitor of prostatic acid phosphatase was designed that is likely to function as a mechanism-based phosphotyrosine phosphatase inactivator. This molecule, 4-(fluoromethyl)phenyl phosphate, represents a useful paradigm for the design of potent and specific phosphatase inhibitors.

Many biological processes, such as signal transduction, nucleic acid repair and synthesis, phospholipid metabolism, and energy storage, involve the formation and cleavage of phosphate ester bonds. Protein phosphorylation in particular is an important mechanism for the regulation of cellular activity (*1*, *2*). In vivo, the steady-state level of protein phosphorylation is controlled by the opposing activities of protein kinases and protein phosphatases. Overexpression of protein tyrosine kinase activity can cause cell transformation (*3*), and it has been suggested that phosphotyrosine phosphatases (PTPases) may function as tumor suppressor genes (*4*). PTPases also exhibit less benign functions. The PTPase YOP 2b is an essential virulence determinant of the bacterium *Yersinia pestis*, the pathogen responsible for the bubonic plague (*5*). Another PTPase is encoded by vaccinia virus and thus may play a role in the pathogenesis of smallpox (*6*).

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Although there is much interest in PTPase inhibitors, efforts to rationally design such inhibitors have been hampered by our limited knowledge of the structural features of these enzymes. We have avoided this problem by designing a mechanism-based inactivator (also known as a suicide inhibitor). Mechanism-based inhibitors are enzyme substrates that undergo an enzyme-catalyzed transformation to give reactive intermediates that, before their release, inactivate the enzyme by forming a covalent bond to an active site residue (*7*). Such molecules are valuable because they are usually potent and specific inhibitors and because they can be used to introduce a radioactive label into the active site of the enzyme.

We used human prostatic acid phosphatase (PAP) as a model enzyme. Although PAP has a broad substrate specificity in vitro, it displays a preference for aryl phosphates (*8*). This phosphatase is an important diagnostic marker for prostate cancer (*9*), and there is circumstantial evidence that it may be involved in the regulation of androgen receptor activity in prostate cells (*10*).

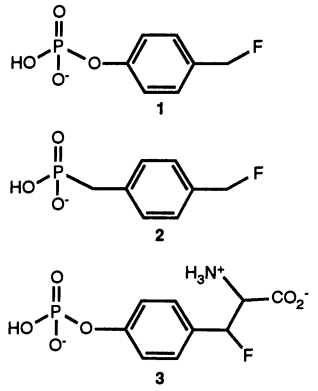

Fig. 1. Structures of phosphatase inhibitors: FMPP (**1**), phosphonate analog (**2**), and β-fluorophosphotyrosine (**3**).

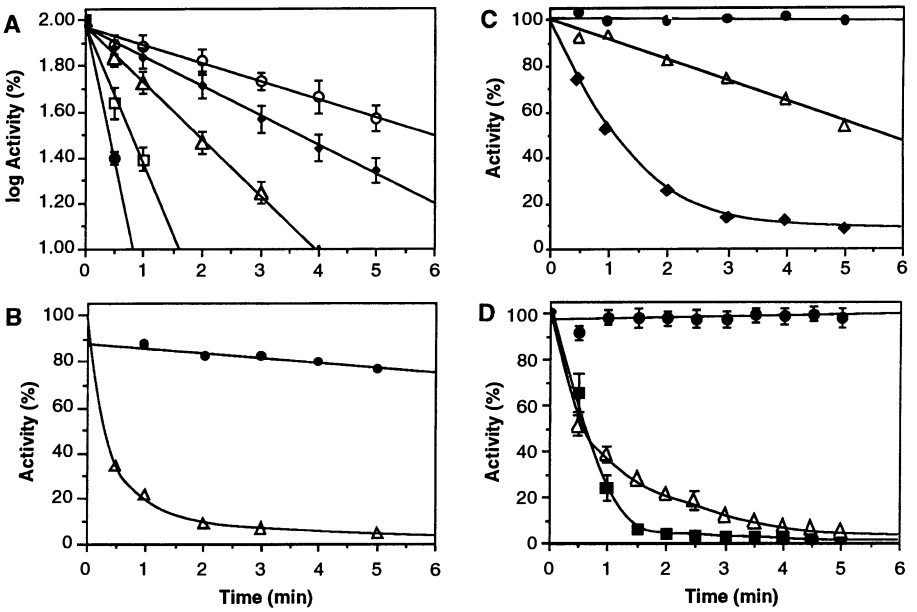

Fig. 2. Inactivation of PAP with FMPP (**1**). We incubated FMPP with PAP (2 μg/ml) in 100 mM sodium acetate buffer (pH 5.0) at 25°C and determined the residual PAP activity by removing 20-μl samples and adding them to 1 ml of 3 mM *p*-nitrophenyl phosphate (PNPP) in 100 mM sodium acetate buffer (pH 5.0). After 5 min in PNPP, the reactions were quenched with 100 μl of 1.25 N NaOH, and the concentration of *p*-nitrophenoxide was determined from the absorbance at 405 nm with an extinction coefficient of 18,000 M^{-1} cm^{-1}. (**A**) Residual PAP activity after incubation with the following concentrations of FMPP: 5.0 mM (filled circles); 500 μM (open squares); 125 μM (open triangles); 70 μM (filled diamonds); and 50 μM (open circles). Error bars represent the standard error of the mean for three measurements. (**B**) Residual PAP activity after treatment with 6.6 mM FMPP (open triangles) or 23 mM phosphonate (**2**) (15K_i) (closed circles). Some residual competitive inhibition is observed with high concentration of **2**, which accounts for the non-100% intercept. (**C**) Residual PAP activity after incubation with 2.3 mM sodium phosphate (filled circles), with sodium phosphate and 190 μM FMPP (open triangles), and with FMPP alone (filled diamonds). (**D**) Residual PAP activity after incubation with 20 mM cysteine (filled circles), with cysteine and 2.3 mM FMPP (filled squares), and with FMPP alone (open triangles). Error bars represent the standard deviation of three measurements.

To design a mechanism-based inactivator of a PTPase, we first identified a chemical motif that has been used for the mechanism-based inhibition of other hydrolytic enzymes, such as peptidases (esterases) and glycosidases (*11*). Compounds containing this motif generate a 4-hydroxybenzylic halide during the course of substrate hydrolysis. This intermediate can undergo the rapid elimination of halide to give a quinone methide, a powerful alkylating agent, in the active site of the enzyme. The enzyme is then inactivated when an active site nucleophile forms a covalent bond to the quinone methide. We chose to test 4-(fluoromethyl)phenyl phosphate (FMPP) (**1**) (Fig. 1), as an inhibitor of PAP because it was the simplest molecule that both contained this motif and would be a good substrate for a PTPase. We predicted that PAP-catalyzed hydrolysis of FMPP would give rise to an intermediate that would undergo a rapid chemical rearrangement to a highly reactive, enzyme-bound quinone methide capable of inactivating the enzyme.

Treatment of purified PAP (*12*) with FMPP resulted in rapid, time-dependent inactivation of the enzyme (Fig. 2). The inactivation process displayed saturation kinetics (inhibition constant K_i = 150 μM), and competitive inhibitors of PAP, inorganic phosphate (Fig. 2C), and tartaric acid (*13*) protect against inactivation. The $t_{1/2}$ for inactivation at saturating levels of FMPP was 35 s. Doubling or halving the PAP concentration did not change the rate of inactivation.

As a possible mechanism for the inactivation process, we suggest that hydrolysis of the phosphate ester bond (rate constant k_1) leads to the formation of a metastable phenol or phenoxide (**4**) at the active site (Fig. 3A). This phenol can partition off the enzyme (k_{off1}) or may suffer the elimination of fluoride ion (k_{elim}) to give a quinone methide (**5**). The quinone methide can inactivate the enzyme (k_{alk}) or can be released into solution (k_{off2}). It is unclear whether PAP remains phosphorylated. If it does, then this inactivation process would provide a simple method for confirming the nature of the enzyme nucleophile (*14*) in the active site.

Because PAP generates a quinone methide each time it turns over, the enzyme inactivation may occur by metabolic inactivation (release of an electrophile to solution followed by alkylation of the enzyme from solution). Such a process would obviate much of the utility of these molecules as specific inhibitors and agents for active site mapping. To test this possibility, we inactivated the enzyme in the presence of nucleophilic scavenging agents. The rate of inactivation was not slowed by the nucleophiles dithiothreitol (5 mM) (*13*), sodium azide (1 mM) (*13*), or cysteine (20 mM) (Fig. 2D). Even the presence of 50 mM sodium azide did not stop the inactivation (*15*). These results suggest that inactivation of the enzyme takes place before the alkylating agent leaves the active site. If the reactive species had left the active site and then alkylated the enzyme at some distal but critical residue, it most likely would have been intercepted by the nucleophilic scavengers, with a consequent reduction in the enzyme inactivation rate.

The potency of the inactivation caused by suicide inhibitors such as FMPP is a function of two ratios, k_{off1}:k_{elim} and k_{off2}:k_{alk} (Fig. 3A). If either ratio is large, then inactivation is unlikely to occur, and vice versa. Aryl phosphates with poor leaving groups, such as the benzylic acetate (**6**), are expected to have slow relative rates of k_{elim} and therefore to inactivate the enzyme slowly if at all. In fact, although benzylic acetate (**6**) is a substrate for PAP, it did not inactivate the enzyme. The time course of the PAP-catalyzed hydrolysis of benzylic acetate (**6**) (monitored by ^1H nuclear magnetic resonance spectroscopy) (*13*) showed the rapid formation of an intermediate, presumably the dephosphorylated phenol (**7**). This intermediate had a $t_{1/2}$ of ~6 hours and decomposed to the *p*-hydroxybenzyl alcohol (**8**), most likely through a quinone methide (Fig. 3B). The fact that the enzyme shows little inactivation during this process (<15%) provides additional evidence that the enzyme is being inactivated through an active site–directed event and not by adventitious alkylation of the enzyme by quinone methide in solution.

To test whether FMPP inactivated PAP

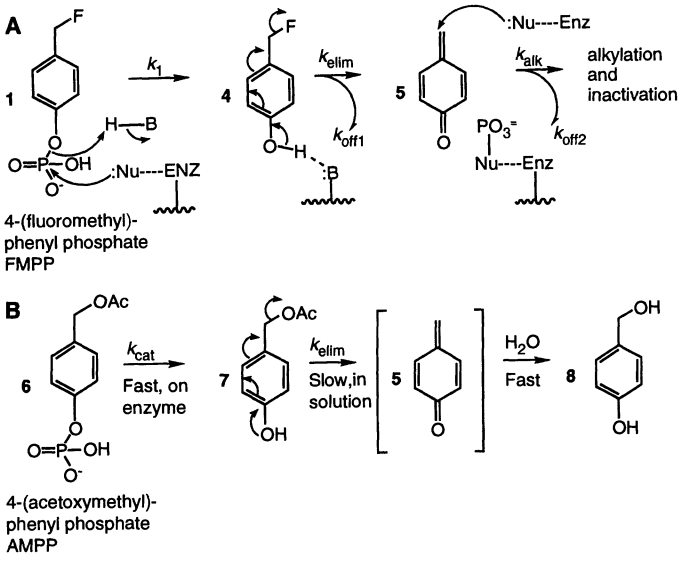

Fig. 3. Proposed mechanism of FMPP and AMPP hydrolysis and PAP inactivation. (**A**) Proposed mechanism of PAP inactivation by FMPP. Hydrolysis of phosphate esters by PAP is thought to proceed by attack on the phosphoros by an active site nucleophile (:Nu-ENZ). The leaving group alcohol is presumably 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.

REFERENCES AND NOTES

1. J. R. Glenney Jr., *Biochim. Biophys. Acta* **1134**, 113 (1992).
2. E. H. Fischer *et al.*, *Science* **253**, 401 (1991).
3. Y. Yarden and A. Ullrich, *Annu. Rev. Biochem.* **57**, 443 (1988).
4. D. R. Alexander, *New Biol.* **2**, 1049 (1990).
5. K. Guan and J. E. Dixon, *Science* **249**, 553 (1990); J. B. Bliska, K. Guan, J. E. Dixon, S. Falkow, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1187 (1991).
6. J. C. Clemens, K. Guan, J. B. Bliska, S. Falkow, J. E. Dixon, *Mol. Microbiol.* **5**, 2617 (1991).
7. R. Silverman, *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology* (CRC Press, Boca Raton, FL, 1988).
8. M. Lin and G. M. Clinton, *Adv. Protein Phosphatases* **4**, 199 (1987); L. Nguyen, A. Chapdelaine, S. Chevalier, *Clin. Chem.* **36**, 1450 (1990).
9. T. M. Chu *et al.*, *Clin. Biochem. Anal.* **11**, 117 (1982); J. E. Heller, *J. Urol.* **137**, 1091 (1987).
10. J. Le Goff, P. Martin, J. Raynaud, *Endocrinology* **123**, 1693 (1988).
11. S. Halazy, V. Berges, A. Ehrhard, C. Danzin, *Bioorg. Chem.* **18**, 330 (1990), M. Wakselman, *Nouv. J. Chim.*, **7**, 439 (1983).
12. R. L. Van Etten and M. S. Saini, *Clin. Chem.* **24**, 1525 (1978).
13. Data not shown.
14. R. L. Van Etten, *Ann. N.Y. Acad. Sci.* **390**, 27 (1982).
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.

16. We thank J. Richardson for helpful discussions. Supported by grants from the National Institutes of Health (R01 GM47918-01) and the American Cancer Society (BE-165).

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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