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A SURFACE *p*-NITROPHENYL PHOSPHATASE OF FROG GASTRIC MUCOSA

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SUMMARY

The isolated frog gastric mucosa catalyzes the hydrolysis of *p*-nitrophenyl phosphate, whether instilled on the nutrient or secretory surface. The *p*-nitrophenyl phosphatase activity, with the substrate on the secretory surface, is increased significantly when acid secretion is stimulated by histamine, and it is inhibited when acid secretion is inhibited by anoxia, ouabain, fluoride, or withdrawal of K^+ from the nutrient solution. This enzymatic activity seems to reflect corresponding changes in the area of apical membranes of oxyntic cells.

INTRODUCTION

Various observations accumulated since the work of Quastel[1] indicate that the intact cell surface can serve an enzymatic function. For example, living yeast cells were found to promote the hydrolysis of exogenous ATP (ref. 2). Since ^{32}P-labelled phosphate split from ATP did not equilibrate with intracellular phosphate, the reaction was presumed to occur on the cell surface.

Surface phosphatases are of special interest in view of the probable role of membrane ATPases in active transport. Cross *et al.*[3] found that intact pulmonary alveolar macrophages exhibit an ATPase stimulated jointly by Na^+ and K^+, similar to the membrane-bound ATPase they obtained from homogenates of these cells. Membrane fractions derived from rabbit and bullfrog gastric mucosae catalyze the hydrolysis of ATP, *p*-nitrophenyl phosphate and other phosphate compounds[4].

The results of the present study show that the intact gastric mucosa functions as a *p*-nitrophenyl phosphatase. This enzymatic activity correlates with acid secretion under certain circumstances.

METHODS

Intact mucosa

After the bullfrog *(Rana catesbeiana)* was pithed, the stomach was removed

and the gastric mucosa freed of its outer smooth muscle. The mucosa was divided longitudinally into halves which were mounted in pairs of plastic chambers; the secretory area exposed was 2.85 cm^2 and the volume of each chamber, 14 ml. The nutrient solution (bathing the serosal surface) contained, in mM: 89.4 NaCl, 18 $NaHCO_3$, 4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$ and 11 glucose; the secretory solution (on the mucosal surface) was 120 mM NaCl. Nutrient and secretory solutions were oxygenated with O_2–CO_2 (95:5, v/v) and 100% O_2, respectively, and contained penicillin (143 units/ml) and streptomycin (0.1 mg/ml). Unless the experiment required a resting mucosa, acid secretion was stimulated with 10^{-4} M histamine·2 HCl in the nutrient solution. The secretory rate was followed by maintaining the secretory solution at pH 7.7 with automatic buret and pH stat (Radiometer ABU II and TTT 1c). Nutrient and secretory solutions were changed after the first hour, and the mucosa short-circuited from then until the end of the experiment.

After the viability of the mucosa was established in the first hour (secretory rate $>$ 4.5 μequiv H^+/h), *p*-nitrophenyl phosphate was added to the nutrient or secretory solution. To estimate its rate of hydrolysis, initial samples of both solutions were taken after 15 min, and final samples, after 75 min. The solutions were then renewed to limit buildup of *p*-nitrophenyl and the cycle was repeated, except that initial samples were taken after 5 min and final ones after 65 min. The *p*-nitrophenyl produced was measured by diluting the sample 1:1 with 0.04 M NaOH, reading at 405 nm in a spectrophotometer, and comparing with a standard *p*-nitrophenyl solution. When required, the amount of P_i in the sample was measured by the method of Lowry and Lopez[5].

Homogenate and membrane fraction

For some experiments, the fresh gastric mucosa was scraped with a scalpel to free the glandular portion from the muscularis and connective tissue. The scrapings were homogenized with glass homogenizer and teflon pestle in 0.25 M sucrose, buffered to pH 7.5 with 5 mM Tris–HCl. The homogenate was centrifuged at 10 000 $\times$ *g* for 10 min, the sediment twice resuspended and respun as before, and the pooled supernatant fluid spun at 37 000 $\times$ *g* for 1 h. The sediment thus obtained was resuspended and frozen for use later as a membrane fraction.

To assay *p*-nitrophenyl phosphatase *in vitro*, the reaction mixture contained 50 mM Tris–HCl at pH 8.5, 2.5 mM $MgCl_2$, 2.5 mM *p*-nitrophenyl phosphate and homogenate or membrane fraction in a final volume of 1 ml. The reaction was carried out at 25 °C. Aliquots were taken at 1 and 11 min after addition of enzyme, diluted with 0.04 M NaOH and read at 405 nm. Protein was determined by the method of Lowry *et al.*[6].

Statistical treatment

Since the *p*-nitrophenyl phosphatase varied considerably among different mucosae, each mucosa was used as its own control for tests of significance. $P < 0.05$ from the *t*-test for paired differences was accepted as indicating a significant difference between test and control periods.

RESULTS

Properties of p-nitrophenyl phosphatase of intact mucosa

In a group of histamine-stimulated mucosae, *p*-nitrophenyl phosphate was added to either secretory or nutrient solutions in a final concentration of 0.5 mM. The products of hydrolysis appeared in both solutions, but the larger amount appeared on the side containing *p*-nitrophenyl phosphate. Fig. 1 shows the total *p*-nitrophenyl liberated during control experiments when *p*-nitrophenyl phosphate was placed in either solution. In neither case did *p*-nitrophenyl phosphatase activity vary significantly over the interval of 4 h. During this time acid secretion dropped slightly. In the rest of the studies to be reported, *p*-nitrophenyl phosphate was added to the secretory solution.

Hydrolysis of *p*-nitrophenyl phosphate, if it occurs on the surface, should liberate P_i in the bathing solutions. Table I gives the results for a group of experiments in which solutions were analyzed for both *p*-nitrophenyl and P_i. It shows that hydrolysis of *p*-nitrophenyl phosphate yielded more P_i than *p*-nitrophenyl in the external solutions. Part of the discrepancy is due to the release of P_i in control periods

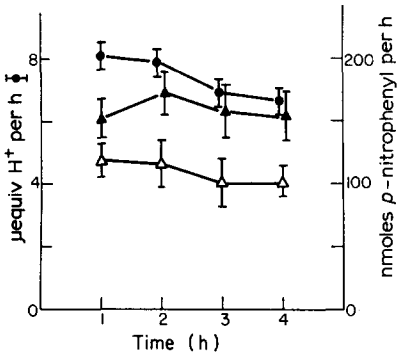


Fig. 1. Time course of acid secretion and *p*-nitrophenyl phosphatase in histamine-stimulated gastric mucosae. The substrate, 0.5 mM *p*-nitrophenyl phosphate, was instilled hourly over 4 h in either secretory solution in nine experiments, or in nutrient solution in six experiments. Means ± S.E. are plotted. ▲—▲, *p*-nitrophenyl phosphate in secretory solution; △—△, *p*-nitrophenyl phosphate in nutrient solution.

TABLE I

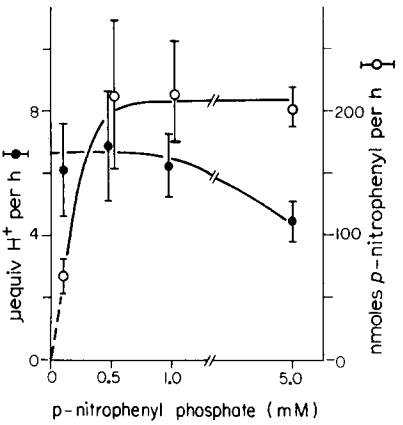
INORGANIC PHOSPHATE AND *p*-NITROPHENYL LIBERATED DURING HYDROLYSIS OF *p*-NITROPHENYL PHOSPHATE BY INTACT MUCOSAE

In a group of four mucosae, nutrient and secretory solutions were analyzed for P_i and *p*-nitrophenyl before, during, and after exposure to 0.5 mM *p*-nitrophenyl phosphate in the secretory solution. Each period was 1 h in duration. Control data are the means of periods before and after.

	product (nmoles/h)	
	P_i	*p-nitrophenyl*
A: 0.5 mM *p*-nitrophenyl phosphate in secretory	342 ± 73	210 ± 41
B: control	68 ± 12	9 ± 1
A−B	274	201

(without *p*-nitrophenyl phosphate); in addition some *p*-nitrophenyl undoubtedly remains dissolved in the mucosa.

The *p*-nitrophenyl phosphatase of the intact mucosa increased and reached a plateau as [*p*-nitrophenyl phosphate] was increased, as illustrated in Fig. 2. Acid secretion and short-circuit current (not shown here) were inhibited at the largest concentration used, 5 mM *p*-nitrophenyl phosphate. The inhibition is probably due to the accumulation of *p*-nitrophenyl in the tissue[7].



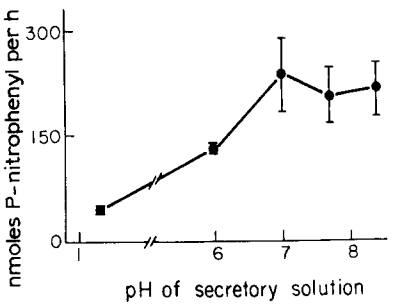


Fig. 2. Dependence of acid secretion and *p*-nitrophenyl phosphatase on concentration of *p*-nitrophenyl phosphate in secretory solution. Composite of seven mucosae, with $n = 3$ at each point.

Fig. 3. Dependence of *p*-nitrophenyl phosphatase on pH of secretory solution, with 0.5 mM *p*-nitrophenyl phosphate in secretory solution. Composite of nine mucosae, with 4–7 measurements at each point.

Fig. 3 shows the effects on *p*-nitrophenyl phosphatase of varying the pH of the secretory solution. The activity did not change substantially between pH 6 and 8, much as Forte *et al.*[4] found for the K-stimulated *p*-nitrophenyl phosphatase of bullfrog gastric microsomes. The low *p*-nitrophenyl phosphatase activity at pH 1.3 rules out the possibility of non-enzymatic acid hydrolysis.

Addition of either Mg^{2+} (1 mM) or K^+ (25 mM) to the secretory solution containing 0.5 mM *p*-nitrophenyl phosphate had no effect on *p*-nitrophenyl phosphatase activity.

Stimulation and inhibition of intact mucosae

The relationship of the *p*-nitrophenyl phosphatase activity to acid secretion was tested by several approaches.

In a group of five experiments, exogenous secretagogue was withheld from the nutrient solution until the secretory rate had declined to a resting level[8]. Stimulation with 10^{-4} M histamine then yielded a 4-fold increase in acid secretion, as shown in Fig. 4. At the same time, *p*-nitrophenyl phosphatase activity increased significantly ($P < 0.01$), but its percentage change was less than that in secretory rate.

Anoxia, which inhibits acid secretion, also inhibited *p*-nitrophenyl phosphatase activity significantly ($P < 0.05$), as illustrated in Fig. 5. The inhibition was reversible

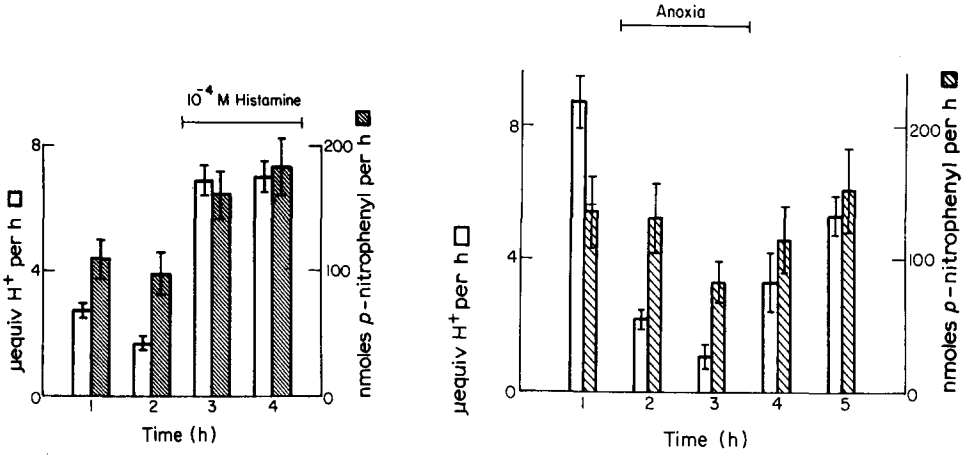


Fig. 4. Effects of histamine addition on acid secretion and *p*-nitrophenyl phosphatase, with 0.5 mM *p*-nitrophenyl phosphate in secretory solution: $n = 5$.

Fig. 5. Effects of anoxia and reoxygenation on acid secretion and *p*-nitrophenyl phosphatase in histamine-stimulated mucosae, with 0.5 mM *p*-nitrophenyl phosphate in secretory solution: $n = 6$.

for both acid secretion and enzyme activity. As noted above, changes in *p*-nitrophenyl phosphatase were proportionately less than those in secretory rate.

Other modes of inhibiting acid secretion also inhibited *p*-nitrophenyl phosphatase activity. Table II shows the effects of withdrawing K^+ from the nutrient solution, as well as the effects of adding ouabain (10^{-4} M), NaF (10^{-2} M), or NaSCN (10^{-2} M) to the nutrient solution. When the *p*-nitrophenyl phosphatase activity during inhibition was compared to that during the control period, inhibition proved to be significant for K^+ withdrawal and treatment with NaF or ouabain, but not for NaSCN addition.

Several phosphate compounds have been shown to inhibit *p*-nitrophenyl phosphatase activity of rabbit gastric microsomes[4]. Some of these were tested in the intact mucosa. The *p*-nitrophenyl phosphatase was measured before, during, and

TABLE II

EFFECTS OF INHIBITORS ON ACID SECRETION AND *p*-NITROPHENYL PHOSPHATASE IN INTACT MUCOSAE

The ratios given are means ± S.E. of the test rate during inhibition divided by the control rate. The latter was measured during the first hour of instillation of 0.5 mM *p*-nitrophenyl phosphate in the secretory solution; the test rate was measured during the second hour of inhibition, except in the case of ouabain, for which the third hour of inhibition was used. The paired difference between control and test rates of *p*-nitrophenyl phosphatase was used to calculate *P*. The number of mucosae in each case is in parentheses.

Mode of inhibition	*Test secretory rate* / *Control secretory rate*	*Test p-nitrophenyl phosphatase* / *Control p-nitrophenyl phosphatase*	*P*
K^+ withdrawal (6)	0.26 ± 0.04	0.65 ± 0.04	< 0.005
10^{-4} M ouabain (4)	0.14 ± 0.04	0.56 ± 0.11	< 0.005
10^{-2} M NaF (5)	0.30 ± 0.08	0.62 ± 0.10	< 0.01
10^{-2} M NaSCN (5)	0.26 ± 0.02	0.85 ± 0.06	N.S.

after exposure to 1 mM of the phosphate compound in the secretory solution. Table III indicates that all the phosphate compounds studied inhibit the *p*-nitrophenyl phosphatase; the order of efficacy resembled that found by Forte *et al.*[4] (with the exception of AMP). Inhibition of *p*-nitrophenyl phosphatase was readily reversible in all experiments. Effects of the phosphate compounds on acid secretion were minor or non-existent.

TABLE III

EFFECTS OF VARIOUS PHOSPHATE COMPOUNDS ON ACID SECRETION AND *p*-NITROPHENYL PHOSPHATASE OF INTACT MUCOSAE

With 0.5 mM *p*-nitrophenyl phosphate in the secretory solution throughout the experiment, acid secretion (second column) and *p*-nitrophenyl phosphatase (third column) were measured for hourly intervals before, during, and after inclusion of 1 mM phosphate compound (first column) in the secretory solution. The data are given in percent of control levels before and after. Each compound was tested in two mucosae. The fourth column gives the estimated inhibition (in percent) of microsomal *p*-nitrophenyl phosphatase from Forte *et al.*[4].

Phosphate compound	*Acid secretion*	*Intact-tissue p-nitrophenyl phosphatase*	*Microsomal p-nitrophenyl phosphatase (ref. 4)*
ATP	91	29	13
ADP	100	28	20
AMP	95	55	98
P_i	90	53	60

p-Nitrophenyl phosphatase in vitro

Direct assay of the K-stimulated *p*-nitrophenyl phosphatase activity for two preparations of whole homogenate gave a mean activity of 66 μmoles *p*-nitrophenyl released per h per gram wet mucosa. The surface activity estimated from Figs 1–3 is 0.2 μmoles/h for a mucosa as mounted, weighing about 0.2 g. Accordingly the surface activity is only about 2% of the total K-stimulated activity of the tissue.

Some properties of the *p*-nitrophenyl phosphatase of the membrane fraction (*cf.* Methods) are presented in Table IV. Stimulation by K^+ was considerable at pH 8.5. Although the enzyme was incubated with 0.05 to 0.1% desoxycholate at pH 7.5 for 30–60 min[9], ouabain failed to inhibit, in agreement with previous findings[4]. Fluoride (10 mM) strongly inhibited the K-stimulated *p*-nitrophenyl phosphatase.

TABLE IV

EFFECTS OF K^+, OUABAIN, AND F^- ON *p*-NITROPHENYL PHOSPHATASE OF MEMBRANE FRACTION FROM FROG GASTRIC MUCOSA

Addition	*No. of assays*	*p-nitrophenyl phosphatase nmoles/min·mg protein*
None	5	8.5
+25 mM K^+	8	38.8
+25 mM K^+ +10^{-3} M ouabain	3	35.6
+25 mM K^+ +10 mM F^-	2	2.2

DISCUSSION

The results show that *p*-nitrophenyl phosphate is hydrolyzed in solutions bathing the isolated gastric mucosa. Provided the concentration of *p*-nitrophenyl phosphate is less than 1 mM, and hydrolysis products are removed at suitable intervals, the procedure does not inhibit acid secretion.

Hydrolysis of *p*-nitrophenyl phosphate generated P_i in external solutions, at least equivalent in amount to the measured *p*-nitrophenyl. Control experiments indicated that this was not due to spontaneous release of P_i from the tissue. It appears, therefore, that hydrolysis occurred in a region easily accessible to the external solutions, at or near the cell surface. Additions of Mg^{2+} and K^+, in concentrations higher than those in the normal nutrient solution, failed to change the *p*-nitrophenyl phosphatase activity of the intact tissue, suggesting that an endogenous supply of these ions is available to the enzyme in this region.

Measurement of the surface enzyme is of interest to the extent that it reflects some physiological activity. A clear correlation of the *p*-nitrophenyl phosphatase with acid secretion was noted when resting mucosae were stimulated with histamine. This could be due to the proliferation of apical membrane of oxyntic cells shown to occur upon stimulation of acid secretion[10–12]. Whether such membrane amplification is due to synthesis of new membrane and enzyme, or to exposure of pre-existing material, is presently unknown.

A group of inhibitors, including anoxia, ouabain, K-free solutions, and NaF, yielded a significant reduction in the *p*-nitrophenyl phosphatase activity of intact mucosae accompanying inhibition of acid secretion. This effect on the enzyme may be due to decreased surface area of the oxyntic cells. The latter has been reported to occur for anoxia[10–11] and K-free solutions[13]. Ouabain probably acts by depleting oxyntic cells of K^+ (ref. 15), as do K-free solutions.

The action of thiocyanate, a familiar inhibitor of acid secretion, is less clear. This agent reduced *p*-nitrophenyl phosphatase activity by 15%, but the inhibition was not significant. Sedar and Forte[14] have reported that thiocyanate affects the ultrastructural appearance of oxyntic cells, but it is not clear from their abstract whether there was actual reduction in surface area.

A comparison of the effects of inhibitors of *p*-nitrophenyl phosphatase *in vitro* (derived from homogenates) with their effects on the intact mucosa is illuminating. Thus NaF almost completely blocked *p*-nitrophenyl phosphatase *in vitro*, and ouabain had no effect, but both agents reduced the intact-tissue *p*-nitrophenyl phosphatase by about 40%. These findings reinforce the conclusion that inhibition of the surface enzyme was indirect, via reduction in secretory area.

The adenine nucleotides and inorganic phosphate inhibit the surface *p*-nitrophenyl phosphatase by a different mode of action. Since these substances did not affect acid secretion, it is unlikely that they decreased secretory area. Instead these agents probably compete directly with *p*-nitrophenyl phosphate at the site of hydrolysis on the cell membrane.

The results of this study do not bear directly upon the question of a possible physiological role for the gastric *p*-nitrophenyl phosphatase. Of interest in this connection is the association of a K-stimulated *p*-nitrophenyl phosphatase with a K-stimulated ATPase in the oxyntic cell microsomes[16]. Independently of its function

in secretion, however, the surface *p*-nitrophenyl phosphatase could prove useful as a dynamic marker for the apical membrane of oxyntic cells.

ACKNOWLEDGEMENT

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