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Properties of the gastric proton pump in unstimulated permeable gastric glands

(permeabilization/digitonin/parietal cell/ATP/ionophore effects)

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ABSTRACT **It was found that digitonin-permeabilized resting gastric glands retained considerable acid-secretory ability. Oligomycin abolished this, and ATP was able to bypass this inhibition and restore acid secretion. Moreover, the effect of anoxia was also bypassed by ATP in these preparations. As in intact glands, acid secretion was K^+ dependent, and the concentration for half-maximal effect was 18.5 ± 1.76 mM in Na^+-free solutions, a value similar to that found for resting intact glands. The slight inhibition of ATP-dependent secretion by either valinomycin or 2,4-dinitrophenol, but total inhibition by a combination of the ionophores, is interpreted to mean that, in resting gastric glands, the *in situ* proton pump is electroneutral and the KCl pathway supplying K^+ to the luminal face of the pump is probably electroneutral.**

Isolation of purified gastric membrane vesicles made it possible to investigate and model the mechanism of H^+ transport by the parietal cell. Historically, the K^+ dependence of acid secretion was recognized in the frog gastric mucosa *in vitro* (1), and the presence of a K^+-activated ATPase was demonstrated (2). The ability of the ATPase to transport H^+ was shown in dog microsomes (3), and the mechanism was revealed to involve H^+-for-K^+ exchange in purified hog gastric vesicles (4). The H^+-pump K^+-leak hypothesis was put forward to explain these observations and has been discussed in several articles (4–6). However, it has proven difficult to explain some of the properties of acid secretion observed in the intact tissue by using the vesicle system. Among these are: the primary energy source for acid secretion (7), the absolute O_2 dependence of acid secretion (8), the apparent electrogenicity of the pump *in vitro* (9), and the increase of resistance with SCN^- inhibition (10). Therefore it was necessary to develop a model intermediate in complexity between the intact cell and isolated vesicles in order to investigate these properties.

One attempt to develop such a model involved permeabilization of parietal cell membranes of isolated rabbit gastric glands by using high-voltage shocks (11). Although evidence was obtained in support of the view that ATP is the primary energy source for acid secretion, electron microscopy of the shocked cells revealed massive mitochondrial damage, indicated by swelling and vacuolation (H. F. Helander, personal communication). This finding raised questions about the interpretation of some of the experiments with shocked glands. Methods were therefore sought that would render the parietal cell membrane permeable without causing gross mitochondrial damage.

The detergent digitonin increases the permeability of various cell types to inorganic ions, metabolites, and enzymes (12, 13). This effect is thought to be due to the interaction of digitonin with cholesterol (14) in the plasma membrane. Mitochondria and endoplasmic reticulum are relatively unaffected (15) because they contain little cholesterol compared to the plasma membrane (16). In the present work, digitonin has been successfully used to permeabilize parietal cells of isolated resting gastric glands of rabbits without affecting the acid-secretory membrane as in shocked glands. Moreover, mitochondrial damage was minimal by morphological and functional criteria. The ATP, O_2, and K^+ dependence of acid secretion were investigated, and it has been possible to examine the electrogenicity of the H^+ pump and the nature of the associated KCl pathway. The results obtained establish digitonin permeabilized gastric glands as a useful model for studying some of the properties of acid secretion by the parietal cell.

MATERIALS AND METHODS

Rabbit gastric glands were prepared as described (17), and the accumulation ratio of the weak base [^{14}C]aminopyrine was used to monitor acid-secretory capacity (18). The glands were washed three times at room temperature in Ca^{2+}-free, high-K^+ medium containing: K^+, 100 mM; Na^+, 31 mM; Mg^{2+}, 1.2 mM; Cl^-, 120 mM; SO_4^{2-}, 1.2 mM; HPO_4^{2-}, 5.0 mM; $H_2PO_4^-$, 1.0 mM; Hepes, 20 mM; Tris, 9.7 mM; phenol red, 10 μg/ml; glucose, 2 mg/ml; rabbit serum albumin, 2 mg/ml. The glands were then incubated in this medium in a shaking water bath at 37°C. O_2 consumption of the glands was measured by using a Warburg respirometer (17).

Permeable gastric glands were prepared by treating a suspension of glands [50 mg (wet weight)/ml] with 20 μg of digitonin per ml of gland suspension at 37°C. Digitonin was dissolved in methanol and added in a minimal volume. Methanol alone had no effect. Leakiness of the cells was monitored by trypan blue uptake (11) and by the appearance of lactate dehydrogenase (LDH) activity in the medium (19).

To determine the K^+ dependence of the secretory response, the solutions were made Na^+ free and the concentration of K^+ was varied. Tetramethylammonium was used as the substituting cation to maintain constant osmolarity. Previous experiments showed that tetramethylammonium is less toxic than choline (20). Ouabain (1 mM) was included in all solutions. K^+ and Na^+ concentrations were checked by flame photometry, using a 443 flame photometer (Instrumentation Laboratory) with external lithium standards for calibration.

To investigate the effects of anoxia, gastric glands were suspended in the glass chamber of a Gilson O_2 electrode apparatus and allowed to consume all the oxygen present in the solution prior to the addition of ATP. The O_2 tension was continuously

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Abbreviations: LDH, lactate dehydrogenase; DNP, 2,4-dinitrophenol.

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monitored to ensure that the glands remained anoxic throughout the experiment.

Digitonin-treated glands were fixed for electron microscopy in 2% (vol/vol) glutaraldehyde in medium for 30–45 min and postfixed for 15 min in 1% osmium tetroxide. After dehydration, the glands were embedded in Spurr embedding medium. Thin sections were prepared and examined in a Phillips 400 microscope.

The metachromatic fluorescence shift of acridine orange was used as described (21) for visual assessment of the cells responding to ATP. The glands were observed with a Zeiss Photomicroscope III.

Oligomycin was dissolved in ethanol, and the ionophores valinomycin and 2,4-dinitrophenol (DNP) were dissolved in methanol. These compounds were always added in minimal volumes and the solvents alone had no effect.

All chemicals were of reagent grade. Collagenase (type I), rabbit albumin, digitonin, oligomycin, valinomycin, ouabain, and ATP, as the disodium salt or Tris salt (for experiments in Na^+-free conditions) were obtained from Sigma; [^{14}C]aminopyrine (84 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear, and DNP was from Fisher.

RESULTS

Effect of Digitonin. When gastric glands were incubated with digitonin at 20 μg/ml, more than 80% of the parietal cells took up trypan blue. The effect of digitonin on aminopyrine accumulation by the glands and on LDH release from the glands was then followed simultaneously.

Gastric glands incubated in high-K^+ medium in the absence of stimuli accumulated aminopyrine to a ratio of about 60 as shown in Fig. 1 (control). The addition of digitonin at 20 μg/ml slowly reduced the aminopyrine accumulation by 50%. However, addition of 5 mM ATP with digitonin to the glands resulted in a transient elevation of the aminopyrine ratio to about 100. ATP alone had little effect, strongly suggesting that digitonin was permeabilizing parietal cells. Although a response to ATP was found, a lower initial aminopyrine ratio was desirable.

Fig. 2 illustrates the effect of oligomycin on aminopyrine accumulation in intact gastric glands incubated for 30 min. At 1 μg/ml, oligomycin lowered the aminopyrine ratio by over 90%. O_2 consumption of the glands was rapidly inhibited by

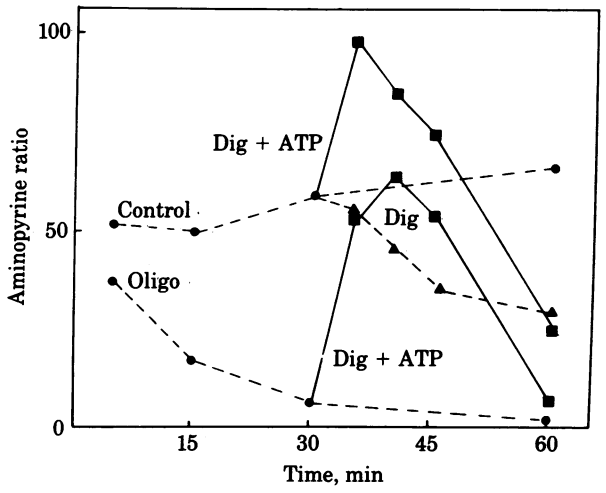

FIG. 1. Effect of digitonin (Dig) alone, or digitonin plus ATP on the aminopyrine accumulation ratio of gastric glands, after 30-min preincubation either without (Control) or with oligomycin (Oligo). Concentrations in this and all subsequent experiments were as follows: oligomycin, 10 μg/ml; digitonin, 20 μg/ml; ATP, 5 mM.

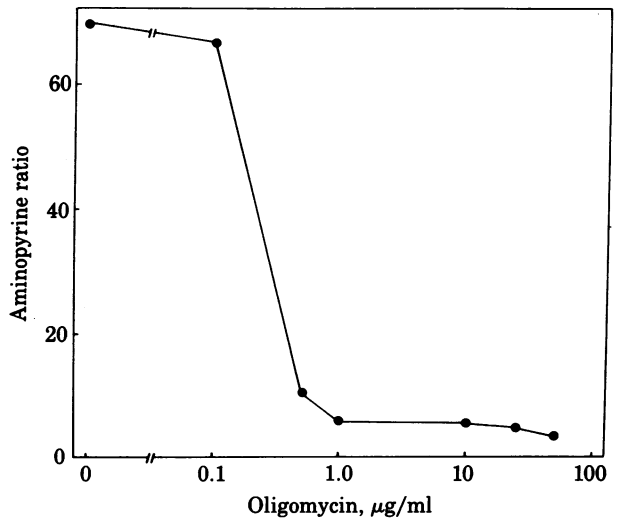

FIG. 2. Effect of oligomycin on the aminopyrine ratio of intact gastric glands incubated for 30 min. Inhibition of aminopyrine accumulation was close to maximum at an oligomycin concentration of 1 μg/ml.

50%, and higher concentrations of oligomycin caused no further inhibition. Therefore, oligomycin, which is known not to affect the gastric H^+,K^+-ATPase (22) was used at 10 μg/ml to pretreat the glands for 30 min. As shown in Fig. 1, the addition of ATP and digitonin then restored the aminopyrine ratio to control levels, an increase of over 10-fold. The effect was transitory, with the maximum increase occurring at 10 min after ATP addition.

The effect of digitonin on LDH release from the gastric glands is shown in Fig. 3. Release of 50–60% of total cellular

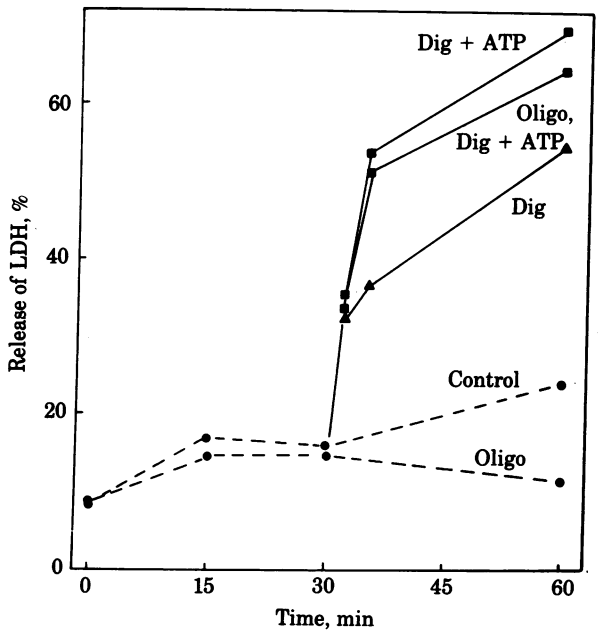

FIG. 3. Effect of digitonin (Dig) on the release of LDH from gastric glands in the same experiment as shown in Fig. 1. Oligomycin (Oligo), when present, was added at time 0; digitonin alone or digitonin + ATP (as indicated) were added at 30 min. Samples were taken in duplicate: one for measurement of aminopyrine accumulation (Fig. 1) and one for assay of LDH. The sample was spun at 10,000 × *g* for 10 sec and the supernatant was rapidly removed. The gland pellet was homogenized in H_2O. LDH was measured in the supernatant and in the pellet homogenate. LDH in the supernatant was then expressed as a percentage of total LDH (supernatant + pellet).

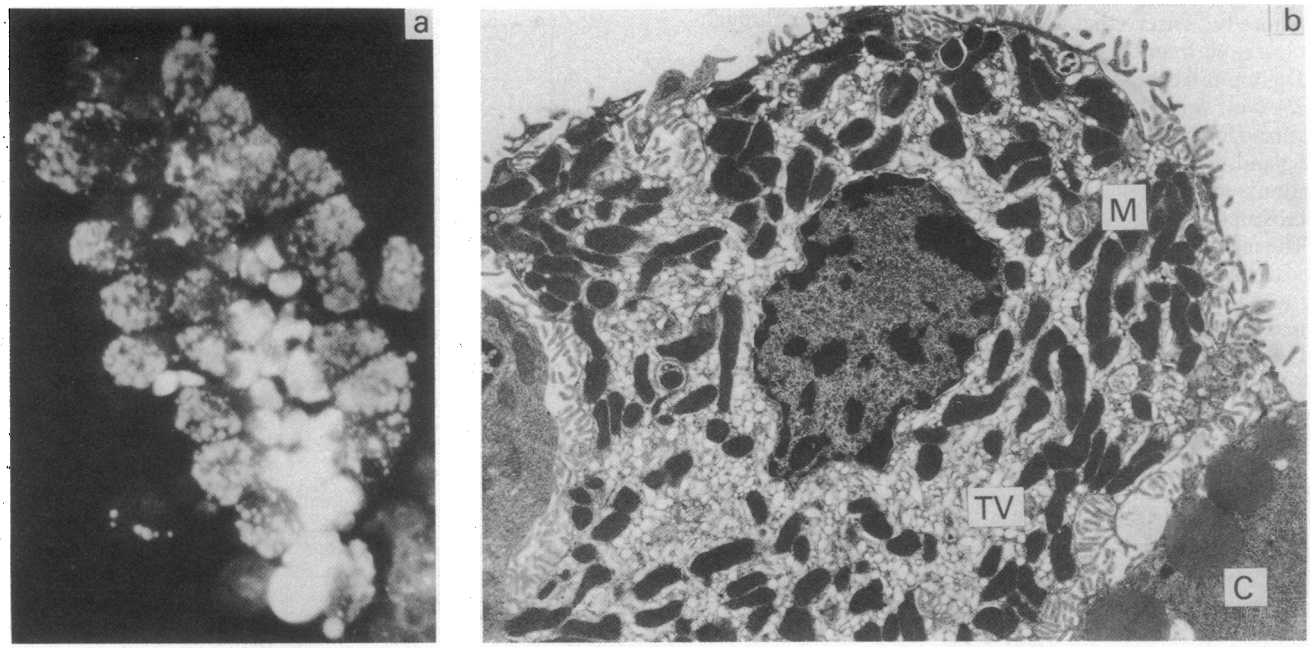

FIG. 4. (*a*) An isolated living gastric gland preincubated for 30 min with oligomycin and digitonin, followed by addition of 5 mM ATP in the presence of 100 μM acridine orange. The white areas are green fluorescence and the grey speckled areas are red fluorescence. Red fluorescence is clearly seen in the parietal cells (around the periphery of the gland), indicating the presence of spaces of low pH. No red fluorescence was observed prior to the addition of ATP. (×175.) (*b*) Electron micrograph of a glandular parietal cell after 30-min preincubation with oligomycin and 10-min incubation with digitonin. The nucleus, mitochondria (M), and tubulovesicles (TV) appear normal, comparable to those of control glands. Chief cells (C), visible in part on either side of the parietal cell, show intact pepsin granules and endoplasmic reticulum. (×3750.)

LDH occurred, confirming that the treated cells were permeable and should admit molecules even larger than ATP.

Microscopic Appearance. When gastric glands were incubated with digitonin at 20 μg/ml with or without oligomycin pretreatment, they appeared morphologically intact as observed by fluorescence microscopy (Fig. 4*a*). Upon addition of ATP, intracellular red patches of acridine orange developed (Fig. 4*a*) as previously described (21). Fig. 4*b* shows the electron microscopic appearance of digitonin-treated gastric glands. No gross morphological changes were seen in the cells. The cellular features of the parietal cells—including the nucleus, mitochondria; and tubulovesicles or secretory canaliculi—appeared intact, comparable to those of untreated control glands.

Requirement for K^+ of the ATP Effect. Varying medium K^+ in the absence of Na^+ showed that the ATP effect is dependent on medium (or cell) K^+ concentrations. It was important to carry out these experiments in the absence of Na^+ because of the known inhibitory effect of cytosolic Na^+ on acid secretion (20). The aminopyrine ratio 10 minutes after ATP addition (at the peak response), at various K^+ concentrations is shown in Fig. 5. The apparent concentration at half-maximal response ($K_{0.5}$) for K^+ is 18.5 ± 1.76 mM ($n = 3$). This is similar to the $K_{0.5}$ found in amphotericin-treated glands in Na^+-free medium (20), indicating that digitonin treatment did not affect the K^+ affinity of the H^+ secretory system. All subsequent experiments were carried out in Na^+-free medium containing 50–60 mM K^+.

Effect of Anoxia. Without added ATP, oligomycin-inhibited digitonin-treated glands showed no measurable aminopyrine accumulation whether in oxygenated or anoxic conditions. In both cases, addition of ATP increased the aminopyrine ratio to about 25, approximately 15-fold (Fig. 6). Hence there was no inhibition of the ATP response by anoxia in this system.

Effect of Ionophores. Initial experiments investigated the effects of valinomycin and DNP on aminopyrine accumulation in intact gastric glands. Both valinomycin (5 μM) and DNP (100 μM) alone completely inhibited aminopyrine accumulation within 30 min, undoubtedly due mainly to mitochondrial uncoupling, but also possibly due to direct effects on the acid-secretory mechanism. Interference from the mitochondrial effects of these ionophores was eliminated by the use of oligomycin-inhibited permeable glands, thus making it possible to investigate any direct effects. Fig. 7 shows that valinomycin and DNP both slightly inhibited (34% and 16%, respectively) the maximum aminopyrine response of oligomycin-inhibited digitonin-treated glands to ATP. Similar results were obtained in medium containing only 20–30 mM K^+. DNP (100 μM) did not cause any inhibition of H^+,K^+-ATPase activity (E. Rabon, personal

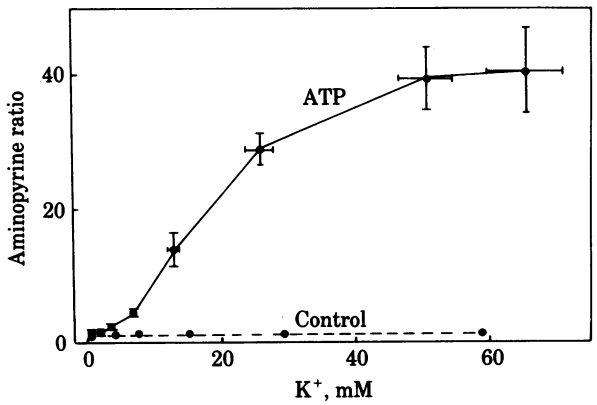

FIG. 5. Effect of varying medium K^+ concentration on aminopyrine accumulation in permeable glands, 10 min after addition of ATP. Glands were washed in Na^+- and K^+-free medium and then preincubated in this medium with oligomycin and digitonin for 20 min. Samples were then rapidly mixed with medium containing various amounts of K^+ and the incubation was continued for a further 10 min to allow complete inhibition by oligomycin. ATP was then added for 10 min. The bars show mean ± SEM, $n = 3$.

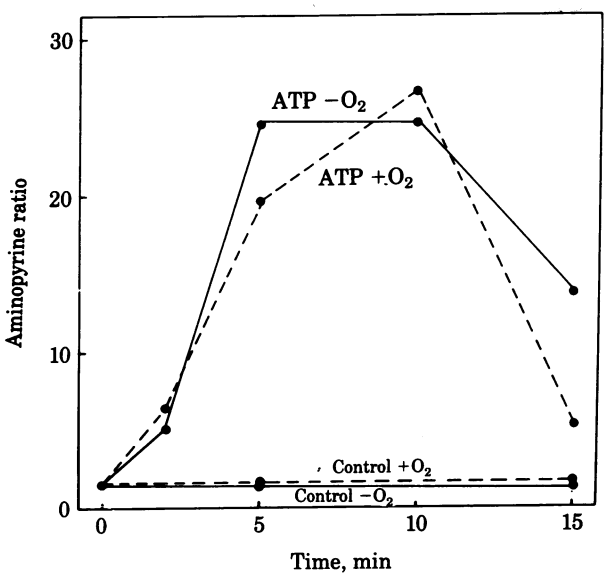

FIG. 6. Effect of ATP, added at time 0, on the aminopyrine ratio in oligomycin-inhibited permeable glands in oxygenated conditions ($+O_2$) and in anoxic conditions ($-O_2$). Glands were pretreated with oligomycin and digitonin in oxygenated conditions for 25 min and then divided into two samples. One sample was made anoxic (3–5 min) and the other was kept in oxygenated conditions. ATP was added to both samples simultaneously. In control samples no ATP was added.

communication). Thus acid secretion is relatively insensitive to these concentrations of the ionophores. However, as expected, valinomycin and DNP together virtually abolished the response. Qualitatively similar effects were observed with the protonophores tetrachlorosalicylanilide and *m*-carbonyl cyanide chlorophenylhydrazone.

DISCUSSION

Uptake of trypan blue and the rapid release of LDH from gastric glands after digitonin treatment indicated that the treated cells were permeable. However, acid secretion was inhibited very slowly, in contrast to the rapid and severe inhibition observed after electrical shock treatment of glands (11). This can be explained by the mitochondrial damage induced by the shocking

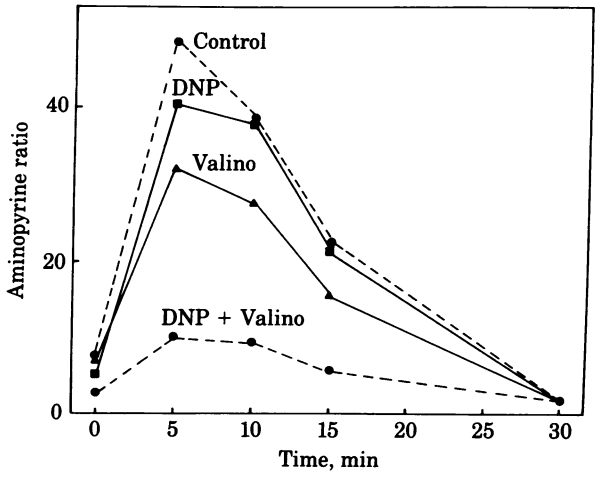

FIG. 7. Effect of valinomycin (5 μM) and DNP (100 μM) on the ATP-supported aminopyrine accumulation of oligomycin-inhibited permeable glands. Glands were preincubated for 30 min with oligomycin alone (Control) or with oligomycin + valinomycin (Valino), DNP, or both. Digitonin and ATP were added together at time 0.

method and the morphological integrity observed in the digitonin preparation. It furthermore supports the view that digitonin does not affect mitochondrial function to any great extent. This maintained mitochondrial function necessitated the use of oligomycin to abolish aminopyrine accumulation prior to digitonin treatment and ATP addition. In both shocked and digitonin-treated glands the secretory membrane remained intact, because the glands accumulated aminopyrine after the addition of ATP.

Oligomycin blocks mitochondrial ATP synthesis and hydrolysis (23), and at the concentration used here it also inhibits the Na^+,K^+-ATPase (24). At the same time, this inhibitor of phosphorylation inhibits tightly coupled respiration. Measurement of O_2 consumption by intact gastric glands showed rapid but only partial inhibition of respiration by oligomycin, although acid secretion was essentially totally abolished. This finding alone argues strongly for a role for ATP in acid secretion and is corroborated by the finding that added ATP restored secretion in oligomycin-inhibited permeable glands. However, these results do not exclude a primary role for a redox system, mitochondrial or extramitochondrial. In shocked glands, ATP was able to restore acid secretion even in the presence of mitochondrial redox inhibitors such as CN^- or N_3^-, an observation that was interpreted to exclude involvement of a mitochondrial redox system in acid secretion. However, the mitochondrial damage observed in shocked parietal cells precludes interpretation of these experiments and furthermore perhaps explains the finding that ATP could not bypass anoxia in shocked glands. In intact glands, inhibition of acid secretion by anoxia can be reversed by reoxygenation (unpublished observations). Alternatively, these results could be explained by involvement of an extramitochondrial redox system in acid secretion. Anoxia must ultimately cause complete blockade of all forms of respiration. The rapidity of its effect on acid secretion suggests that it is unlikely that the tissue contains sufficient quantities of electron acceptors to maintain any form of redox function for very long in the absence of O_2. In digitonin-permeabilized glands, ATP was able to bypass the combination of oligomycin and anoxia and support acid secretion. This result, in our view, establishes the concept that ATP provides the sole energy source for acid secretion. The absolute dependence of acid secretion on the presence of O_2 therefore means that the parietal cell utilizes only mitochondrially generated ATP for acid secretion and that there are no large stores of this nucleotide directly available for H^+ transport. The transitory effect of ATP on acid secretion in permeable glands could be due to several factors, including inhibition of H^+,K^+-ATPase by ADP and permeabilization of the secretory membrane by digitonin after longer incubation. Preliminary experiments with ATP-regenerating systems indicate that inhibition by ADP is possibly involved. However, further investigation is needed.

As found with all other models, the K^+ dependence of acid secretion was confirmed in digitonin-treated glands. The K^+ dose–response curve of ATP-supported acid secretion illustrates the functional integrity of this preparation. This integrity is also supported by the finding that in these permeable glands the $K_{0.5}$ for K^+ in the absence of Na^+, 18.5 ± 1.76 mM is similar to that found in intact glands after amphotericin treatment, 16.5 ± 0.9 mM (20). Because it is known that the gastric H^+,K^+-ATPase possesses both activating and inhibitory cation sites (25), the $K_{0.5}$ as determined here is the combination of K^+ binding to both sites.

By using oligomycin-inhibited permeable glands, it has been possible to investigate the effects of ionophores directly on the acid-secretory mechanism *in situ*, without interference from mitochondrial effects, and thus gain some insight into the elec-

trogenicity of the pump *in vitro* and into the conductive pathways of the luminal or acid-secretory membrane. In the presence of valinomycin and DNP, acid secretion was virtually abolished, indicating that adequate concentrations of both ionophores were present within the luminal membrane.

The small inhibition of ATP-supported acid secretion by the protonophore DNP indicates that the Cl^- conductance is very low. If a large Cl^- conductance were present, addition of an H^+ conductance would cause rapid dissipation of the pH gradient by efflux of HCl. Because DNP is an electrogenic protonophore, if the ATP-driven H^+ transport in permeable glands were due to an electrogenic proton pump, no pH gradient would develop in the presence of adequate concentrations of the protonophore. Therefore it seems very unlikely that the gastric proton pump is electrogenic. Similar conclusions were reached from studies using isolated gastric vesicles (4).

The finding that valinomycin partially inhibits acid secretion in permeable glands (in low- and high-K^+ media) is surprising, because valinomycin increases H^+ transport in gastric vesicles depleted of internal K^+ (4–6). This latter finding has been interpreted to mean that gastric vesicles shown to derive from the secretory membrane (26) are limited in H^+ transport ability by low K^+ permeability. Evidently, the situation is different in permeable glands. At the low pH found in the secretory canaliculus of the parietal cell, the proton-carrying ability of valinomycin (27) might be sufficient to partially dissipate the pH gradient, as observed. Nevertheless, in contrast to isolated vesicles, considerable acid secretion is maintained in permeable glands in high K^+ medium in the absence of ionophore, suggesting that K^+ is amply available at the luminal side of the secretory membrane. Because increasing medium (or cell) K^+ is reflected by an increasing response of ATP-supported acid secretion, it seems necessary to postulate the presence of an adequate KCl or K^+:Cl^- pathway at the secretory membrane in permeable glands. This property may be absent or masked in isolated vesicles or perhaps lost during the isolation procedure. Moreover, the presence of a low Cl^- conductance implies that entry of KCl is unlikely to be by coupling of K^+ and Cl^- conductances. Hence K^+ and Cl^- entry to the luminal surface of the H^+,K^+-ATPase is probably by electroneutral pathways (4, 28). This also correlates with the finding that Cl^- permeability exceeds tissue conductance by a considerable amount (29). Furosemide at 1 mM inhibits secretion by 80% in these permeable glands, whereas diamox is ineffective. This also suggests coupling between K^+ and Cl^- transport at the apical membrane of the unstimulated parietal cell.

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