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The site of acid secretion in the mammalian parietal cell

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Initiation of acid secretion in the gastric mucosa is accompanied by a morphological transformation in which the acid pump, the H^+/K^+-ATPase, translocates from a cytoplasmic vesicular location to the secretory surface lining the canaliculi. Associated with the morphological changes, activation of K^+ and Cl^- pathways are necessary to supply K^+ to the extracytoplasmic face of the pump. Although the pump in the secretory membrane is known to secrete acid, it is not known whether activation of the KCl pathway occurs in the tubulovesicular membrane prior to the formation of the canaliculus, or when the pump is in the secretory membrane. The cellular site of activation of acid secretion in the rabbit gastric parietal cell was investigated using the covalent binding of [^{3}H]omeprazole as a probe of acid secretion in rabbit gastric glands that were undergoing stimulation in vitro. This compound depends on an acidic environment for activation and covalent binding to the H^+/K^+-ATPase. Electron microscopic autoradiography showed that activation of the enzyme occurred only when it was present in the canalicular membrane and not when it was present in the cytoplasmic tubulovesicular membrane. Hence there is likely to be a physical separation of K^+ and/or Cl^- pathways from the ATPase in the resting cell, and stimulation of acid secretion is dependent on colocalization of these pathways in the canalicular membrane.

Introduction

Acid secretion by the stomach is present in all vertebrates, and is subject to regulation. It is the property of oxynticopeptic cells in reptiles and birds, and of the parietal cell in mammals.

In oxynticopeptic cells, the initiation of acid secretion is accompanied by a morphological transformation that results in an apparent expansion of the apical plasma membrane [1]. In the resting mammalian parietal cell, the cytoplasm contains abundant smooth surfaced tubulo-vesicles between 0.1 and 0.2 μm in diameter and small secretory canaliculi. The stimulated parietal cell shows expanded secretory canaliculi with multiple microvilli and a reduced number of tubulovesicles. Morphometry has shown that loss of cytoplasmic tubulo-vesicles in rabbit gastric glands is balanced by this expansion of the secretory canaliculi [2]. Hence there is no change in surface area of the pump membrane itself, only a change in location of the pump. The existence of this morphological transformation was detected at the end of the last century and led to the suggestion that the parietal cell was the cell responsible for acid secretion [3].

The demonstration that uptake of the metachromatic weak base, acridine orange, was into the canaliculus of the parietal cell of rabbit gastric glands provided direct evidence that acid secretion occurred into that structure [4]. Staining of resting and stimulated cells with a monoclonal antibody directed against the H^+/K^+-ATPase, the enzyme responsible for acid secretion [5], showed that the pump was present in the cytoplasmic tubulo-vesicles in the resting cell and in the plasma membrane of the canaliculi in the stimulated cell [6]. Accordingly the morphological transformation associated with stimulation of the parietal cell is accompanied by translocation of the acid pump from a cytoplasmic to a secretory membrane location.

When the H^+/K^+-ATPase is isolated from non-secreting gastric mucosa, most of it is present in inside-out vesicles that represent the cytoplasmic form of the enzyme. To demonstrate K^+ stimulated activity it is necessary to provide a K^+ permeation pathway in these vesicles using ionophores such as valinomycin or nigericin or to preincubate the vesicles in K^+ containing solutions [5]. It is clear therefore that these resting vesicles do not possess a K^+ pathway necessary for supplying K^+ to the extracytoplasmic face of the ATPase. K^+ is required to destabilize the E_2-P form of

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the enzyme [7] and is exchanged for H^+ to result in acid secretion by the enzyme [5]. The difference between resting and secreting pump is the presence of KCl pathways in the pump membrane to allow access of K^+ to the extracytoplasmic face of the pump. When the pump is isolated from a stimulated stomach, a significant amount of it is found in a denser fraction of a density gradient which contains a mixture of membranes including secretory canalicular derived membranes [8]. The pump found in this dense fraction displays acid transport without ionophores, indicating a KCl pathway. Electron diffraction of canalicular as compared to tubulovesicular membranes showed no observable difference in terms of intramembranal protein at 27 Å resolution [9]. The density change is due to maintained association with cytoskeletal proteins such as actin during homogenisation and membrane fractionation of the stimulated parietal cell and not to a change in the density of the pump membrane itself [8]. Thus although the KCl pathway is present in the heavier fraction, these data do not show whether activation of the pump occurs in the cytoplasmic vesicular form along with cytoskeletal events, or only in the canaliculus after translocation has occurred [8].

The question therefore arises as to when the morphological transformation is the KCl pathway activated. Is there activation of a dormant pathway in the cytoplasmic vesicles along with cytoskeletal binding or is the KCl pathway present only when the pump is present in the microvilli of the canaliculus which contain the actin filaments? This problem therefore requires a method of detecting active as compared to inactive pumps in the intact cell where the characteristic morphological features are retained.

Here, we have taken advantage of the properties of the covalent inhibitor of the H^+/K^+-ATPase, omeprazole. This compound is an acid activated weak base ($pK_a = 4$) prodrug [10]. It is therefore accumulated in the acid space of transporting gastric vesicles and the canaliculi of the stimulated parietal cell. In the acid space omeprazole is converted to a relatively membrane impermeant cationic sulfenamide that reacts with two extracytoplasmic cysteines of the α subunit of the H^+/K^+-ATPase, located in or between membrane spanning segments 5 and 6 and between membrane spanning segments 7 and 8 [11] (Fig. 1). The sulfenamide is relatively stable in acid, but extremely unstable at neutral pH ($t_{1/2} < 100$ ms) [10]. Any backleak of the sulfenamide into the cytoplasmic compartment would result in immediate conversion of the sulfenamide into a non-reactive sulfide derivative. Since omeprazole itself is inactive at neutral pH, it will neither bind to nor inactivate resting pump. The ability of omeprazole to be accumulated and acid activated to make a covalent marker for the acid transporting ATPase, makes it a suitable probe for the site of acid

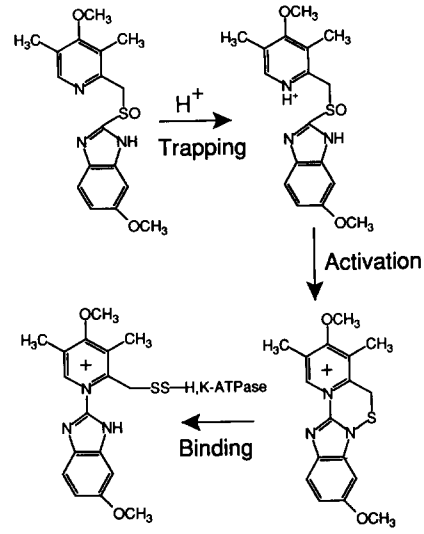

Fig. 1. The mechanism of action of omeprazole. Omeprazole is converted to a sulfenamide in the presence of acid. It then binds to sulfhydryl groups of the H^+/K^+-ATPase.

secretion by the pump. When a rabbit is treated with labeled omeprazole in vivo, label is found in only the α subunit of the H^+/K^+-ATPase and no other protein retains radioactivity [12]. Using electron microscopic autoradiography, it should be possible to determine whether stimulation of the pump in the parietal cell occurs first in the cytoplasmic tubulovesicles or only upon appearance of the pump in the canaliculus.

Rabbit gastric glands are a model of the stomach in vitro [13] which can be made relatively resting or be stimulated to secrete acid. Acid secretion is detected by the uptake of the weak base, [^{14}C]aminopyrine (AP, $pK_a = 5$), usually expressed as a ratio of concentration of weak base in the intracellular space to the concentration of weak base in the extracellular medium (AP ratio). Following stimulation, the accumulation of AP reaches steady state in about 20 min [2,13]. It is not possible to abolish acid secretion by receptor antagonists in this in vitro model, nor is it possible to abolish acid secretion in the in vivo rabbit stomach by receptor antagonists [14].

In the work presented here, rabbit gastric glands were stimulated in the presence of [^{3}H]omeprazole, and the uptake of [^{14}C]aminopyrine and binding of [^{3}H]omeprazole were followed in conjunction with localization of the site of binding by EM autoradiography. It is shown that the increment of bound drug at 5, 10 or 30 min following stimulation is confined largely to the secretory canaliculi. Hence activation of the KCl pathway does not occur until the pump is present in the membrane of that structure.

Methods

Gland preparation

Glands were prepared by enzyme digestion as previously described [13]. Briefly, the rabbit was anes-

thetized with nembutal (50 mg/kg), the gastric arteries perfused with phosphate buffered saline under high pressure, and the mucosa scraped and chopped finely with scissors. Digestion was performed at 37°C, using 20 mg collagenase/50 ml incubation medium (NaCl, 140 mM; $MgSO_4 \cdot 7H_2O$, 1.2 mM; $CaCl_2.2H_2O$, 1 mM; Hepes, 10 mM; KOH, 5.4 mM; cimetidine, 100 μM; D(+)glucose, 0.5 mg/ml; BSA, 2 mg/ml). After 25 min, digestion was determined to be complete by microscopic evaluation. The glands were washed three times with incubation medium and allowed to settle, the medium was removed and the glands were resuspended in incubation medium.

[^{3}H]Omeprazole labeling

Labeling was initiated by the addition of [^{3}H]omeprazole (0.37 Ci/mmol) (1 μCi/ml gland suspension). At the initial time 1 mM dbcAMP (final concentration) was added to stimulate acid secretion in one group of glands, while the unstimulated group received an equal volume of incubation media. Aliquots of the suspension were removed at 0 min, 5 min, 10 min, 30 min and 60 min and fixed with 2% glutaraldehyde. After 1 h of fixation at 37°C in an orbital shaker (150 RPM), 1 ml of the fixed gland suspension was removed from each timed aliquot, and spun in an Eppendorf centrifuge for 5 min. The pellet was washed three times with incubation medium, dried overnight at 60°C then dissolved in 1 M NaOH. The amount of radioactivity for each time point was determined by liquid scintillation counting to measure total [^{3}H] omeprazole remaining in the glands as a function of time. The remaining fixed cell suspension from the various time points was used for EM autoradiography as outlined below.

EM autoradiography

After fixation for an additional 2 h at room temperature the glands were rinsed, post fixed in 1% buffered OsO_4 and embedded in Polybed (Polysciences, Warrington, PA). 100 nm sections were collected on grids, contrasted with uranyl acetate and lead hydroxide and coated with carbon. Using the loop method, a thin layer of Ilford L4 photographic emulsion was then placed on each grid which was stored at 4°C for 1 month.

The emulsion was developed according to standard procedures [15] and the number of silver grains on randomly chosen parietal cells were counted. Only cells sectioned through the nucleus were studied. The silver grains were classified into two groups, those over the secretory canaliculi plus the immediate neighboring cytoplasm (i.e., within 0.5 μm region immediately adjacent to the secretory canaliculi), and those over the cytoplasm (tubulovesicles). At the earlier time points, if activation was to occur in the cytoplasmic compartment, prior to formation of the microvilli, an increase of radioactivity would be detected in this compartment irrespective of the decrease in surface area that might be expected. If activation were occurring exclusively in the canalicular compartment, then the increase of radioactivity at the earlier time points would be found in this compartment and not over the cytoplasmi (less the region immediately adjacent to the canaliculi). Thirty parietal cells were studied at each time point. Cells from stimulated glands examined at 5 and 10 min time points with a total of less than 5 silver grains per cell and cells from the 30 and 60 min time points with a total of less than 10 silver grains per cell were excluded from statistical analysis as they were considered to have been damaged during the preparation and hence unresponsive to cAMP.

Aminopyrine accumulation

In a parallel experiment the AP accumulation was determined in stimulated and unstimulated glands. [^{14}C]Aminopyrine was added to glands in the presence or absence of 1 mM dbcAMP and 1 ml aliquots were removed at 0, 5, 10, 30 and 60 min. The samples were centrifuged briefly, the supernatant removed and the pellet was dried and weighed. The pellet was then dissolved in 1 M NaOH and the amount of radioactivity in the pellet and supernatant was determined by liquid scintillation counting. AP accumulation is expressed as the ratio of [^{14}C]AP in the intracellular versus extracellular water [16].

Results

Stimulation of acid secretion

In the unstimulated gastric glands, there was no increase in the aminopyrine uptake for the 5 and 10 min time points, which remained at a typical resting value of about 20 [16]. There was a small increase at 30 min and a further increase at 60 min reaching an AP ratio of 50. It has not been possible, using receptor antagonists, to reduce acid secretion to zero in this model (which would give an aminopyrine ratio of unity) [16]. This can be accounted for by several factors, such as pump activity in spite of the presence of cimetidine and continuing activation of pumps over the 60 min time course of the experiment (Fig. 2A).

In the presence of dbcAMP, there was little change in the aminopyrine ratio at 5 min, but a noticeable increase at 10 min, which then reached a maximum at 30 min. This time course is relatively typical of the in vitro rabbit gastric gland model under maximal stimulation. The maximal aminopyrine ratio observed was 292 representing a greater than 15-fold stimulation of weak base accumulation. In this particular experiment, at 60 min there was a decline in the ratio by about

30%, but this still represents a large increase over the unstimulated state (Fig. 2A).

Binding of [^{3}H]omeprazole

The accumulation of this weak base is dependent on the presence of acid spaces with a $pH_i < 4.0$, since the pK_a of omeprazole is 4.0. Hence, the acidity has to be 10-fold greater for omeprazole accumulation than for aminopyrine accumulation since the pK_a of aminopyrine is 5.0. Following accumulation, the acid conversion occurs, resulting in covalent binding of the drug to two of the extracytoplasmic SH groups of the H^+/K^+-ATPase. Binding is measured in the experiment illustrated in Fig. 2B, since washing will have removed most of the unbound counts. At 5 min the amount in the glands was very slightly greater in stimulated than in unstimulated glands, about $60 \cdot 10^3$ cpm mg^{-1}, showing that acid secretion was occurring under both conditions, consistent with the aminopyrine ratio results. After 10 min of stimulation, there was a significant increase in bound omeprazole which rose to a steady state level of $320 \cdot 10^3$ cpm mg^{-1} at the 30 min time point, similar to the increase of the aminopyrine ratio.

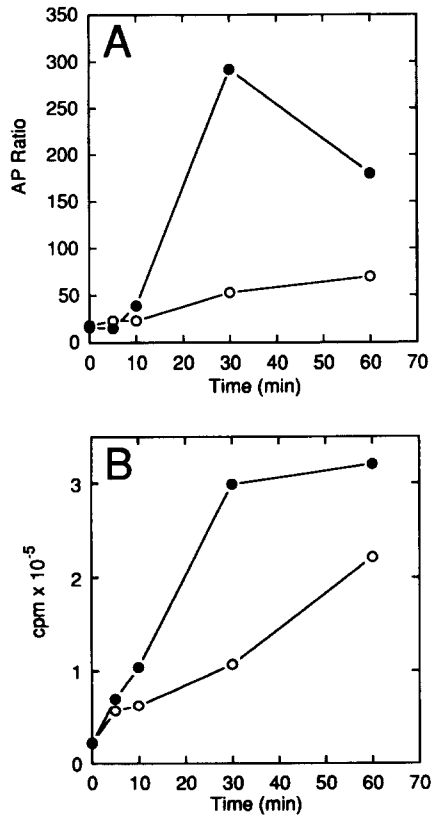

Fig. 2. Time course of AP ratio and [^{3}H]omeprazole binding in unstimulated and stimulated rabbit gastric glands. (A) AP ratio. All glands were incubated in the presence of 100 μM cimetidine. Glands were stimulated with 1 mM dbcAMP. (B) [^{3}H]Omeprazole binding. Glands were washed three times following labeling. cpm values are expressed per mg dry weight of glands. •, stimulated; ○, unstimulated.

In the unstimulated state, there was also a progressive increase in bound omeprazole. This result correlates qualitatively with the accumulation of aminopyrine. It is accounted for by continued activation of a small number of pumps in spite of the continued presence of the H2 antagonist, cimetidine. It is not possible to reduce the aminopyrine ratio to unity with receptor antagonists, as mentioned above. Generating a resting state in the rabbit in vivo is also not achievable using H2 receptor antagonists [14].

Site of binding of omeprazole

The sites of binding of omeprazole were determined using electron microscopic autoradiography to locate the grains. The data obtained are divided into total grains detected and the distribution of the grains between cytoplasm and canaliculus.

Fig. 3 shows the total radioactivity detected over the parietal cells counted during the experimental period in the presence of cimetidine or dbcAMP. The increase in total omeprazole bound detected microscopically corresponds reasonably to that detected when total binding is measured by centrifugation of the labeled glands. Thus there is an increase at 5 min in the stimulated and unstimulated glands, but the increase is larger in the stimulated sample. The increase is maintained at 10 min and there is a large increase in the stimulated, but not the unstimulated glands at 30 min, followed by a slower rise at the 60 min time point.

Fig. 4A and B shows the distribution of omeprazole between cytoplasmic and canalicular compartments as a function of time and of stimulation. In the unstimulated cytoplasmic compartment over the 60 min time period of the experiment, there was a gradual increase in the number of grains up to the 60 min time point. There was also a gradual increase in the canalicular compartment of the unstimulated glands over the 60 min time course of the experiment. The radioactivity in these two compartments remained relatively equal. These data indicate there was an overall increase in omeprazole binding even in unstimulated glands, as was observed in Fig. 2. This is accounted for by the presence of active pumps even in the presence of cimetidine, and continuing activation of pumps over the time course of the experiment.

In the stimulated state, the number and increment of counts over the cytoplasmic compartment is virtually the same as found in the unstimulated state. This could not happen if stimulation of the pump was occurring in the tubulovesicular form. In contrast, the number of grains over the canalicular compartment increased markedly at 5 and 10 min and increased further at 30 min and slightly at 60 min, again reflecting the omeprazole remaining associated with the glands following centrifugation and washing (Fig. 2B). The increase of label at 5 and 10 min is larger than what might be

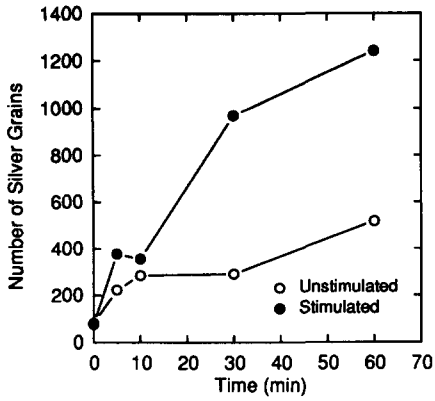

Fig. 3. Time course of the number of silver grains appearing over the parietal cell. The total number of silver grains appearing over both the canaliculi and cytoplasmic compartments were plotted for each time point. Each point represents the sum of silver grains of all cells counted per time increment ($n = 30$).

expected from the aminopyrine ratio. However, the aminopyrine ratio reflects the result of active secretion by the H^+/K^+-ATPase, being dependent on the pH and volume of the acidic compartment. It requires a large number of pumps turning over for some considerable time to significantly affect the AP ratio. However, even acidity at the extracytoplasmic face of a pump that has just been activated is sufficient to activate omeprazole with resultant binding to the ATPase. This lag between pump activation and aminopyrine accumulation in this model has also been shown for the morphological transformation [2].

Typical micrographs of a stimulated and an unstimulated cell at 10 min are presented in Fig. 5A and B. It can be seen that virtually all of the grains in the stimulated cell are present over the canalicular region, whereas the few silver grains in the unstimulated cell are mostly over the cytoplasmic compartment.

Discussion

The stimulation of the parietal cell results in insertion of pump molecules into the canalicular membrane [6] and in activation of a KCl transport system to allow access of K^+ to the luminal side of the pump [8]. Although both events are necessary for net acid output by the gastric gland, it is not known whether activation of the KCl pathway precedes or is simultaneous with the morphological events.

The weak base [^{14}C]aminopyrine is accumulated in the acid space of the active parietal cell [14], as is another weak base, acridine orange. The accumulation of acridine orange allows visualization of the acid space at the light microscopic level [4] which has sufficient

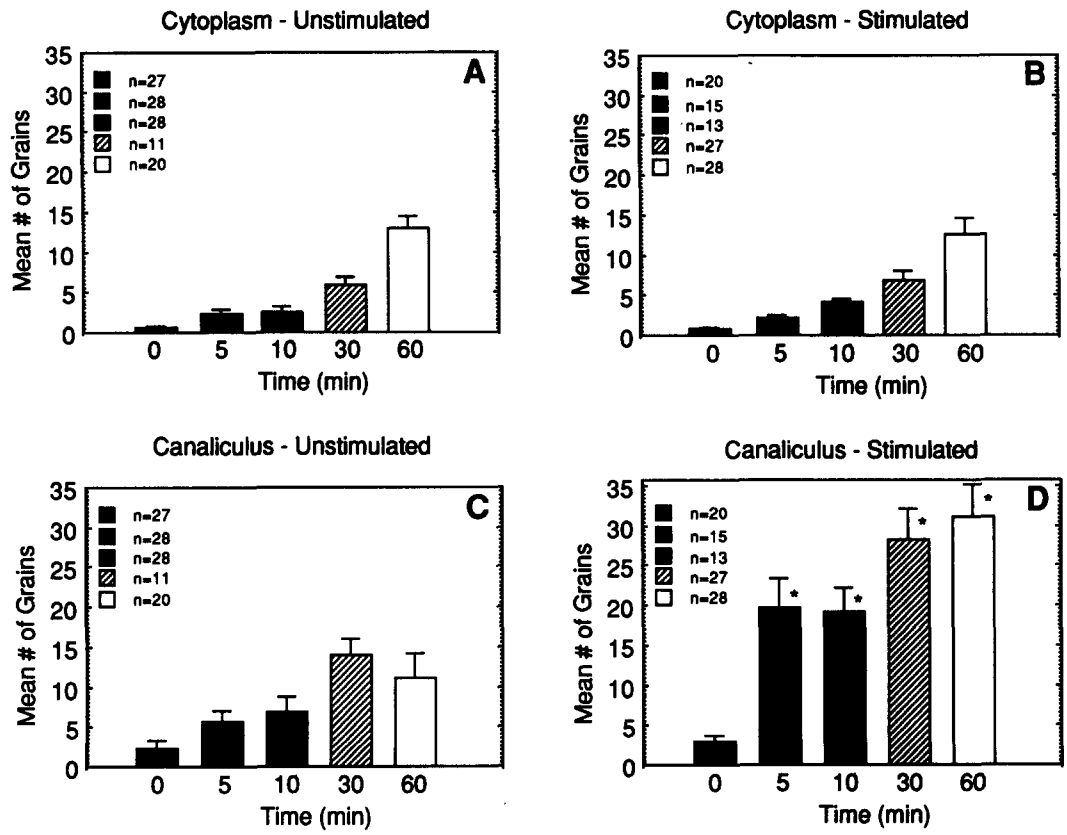

Fig. 4. Time course of the number of silver grains appearing over the cytoplasmic and canalicular compartments of unstimulated and stimulated parietal cells from EM autoradiography. The mean number of grains ± S.E. is plotted for each condition. The difference between the mean number of grains over the canalicular compartment from the stimulated and unstimulated cells at 5, 10, 30 and 60 min is significant ($P \leq 0.05$).

resolution to show secretory canaliculi but not tubulovesicles. This probe allowed a direct demonstration of the site of acid output in the parietal cell, but not the site of initiation of acid secretion.

In this work, we have used the requirement of acid activation of omeprazole and its subsequent covalent binding to the pump [10] to track the site of initiation of acid secretion in rabbit gastric glands. Omeprazole will bind only to pump molecules that are present in the membrane of a compartment that is acidic or to pumps that are generating acid on their extracytoplasmic surface. Following activation there is a diffusion limited reaction of the sulfenamide that is formed (Fig. 1) with available thiols [10]. In acid transporting gastric vesicles, omeprazole binds to the a subunit of the H^+/K^+-ATPase with a stoichiometry of 2 mol omeprazole per mol phosphoenzyme [17,18]. The cysteines that react have been identified as cysteine 813 or 822 and cysteine 892 [11]. Omeprazole will therefore label and allow visualization of active H^+/K^+-ATPase α,β heterodimers.

If activation of the pump was to occur while the pump was still present in the cytoplasm as tubulovesicles, an increase in the number of grains found over the cytoplasm would precede an increase in canalicular counts. Alternatively, if activation of the

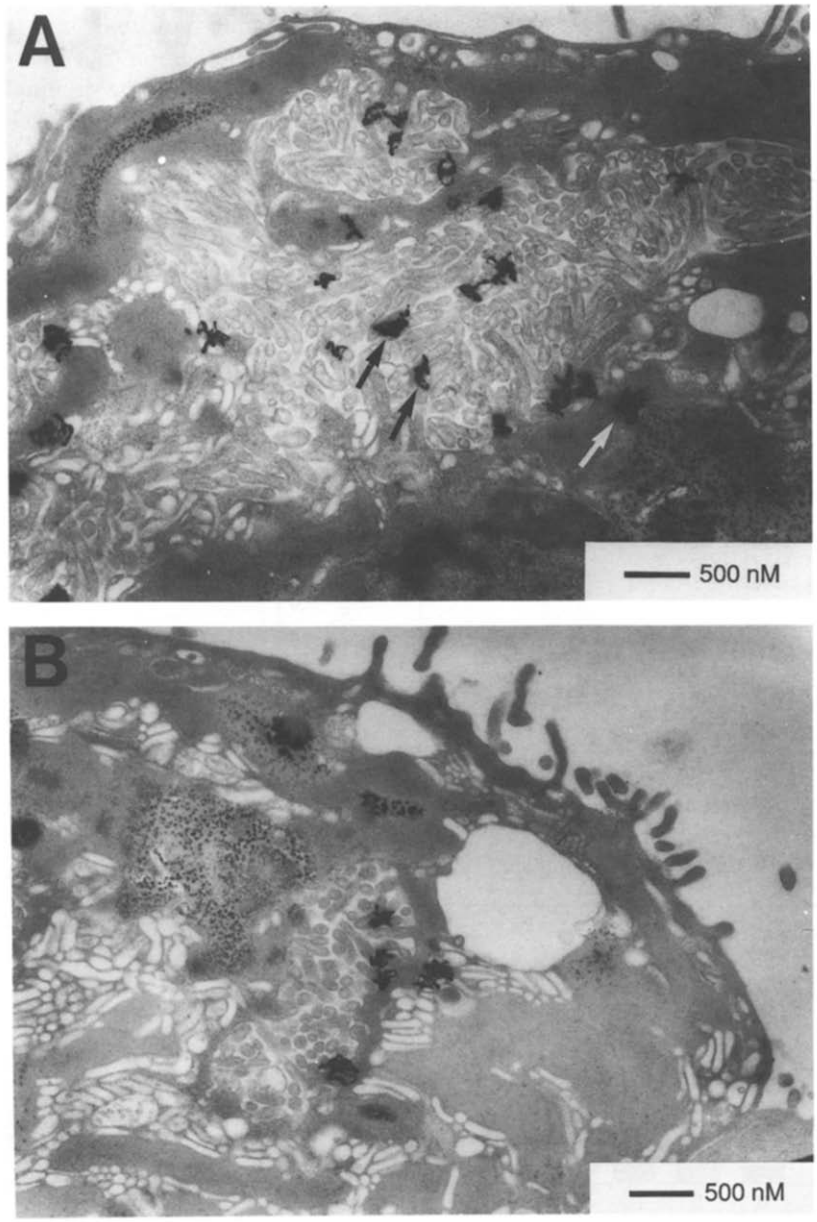

Fig. 5. EM autoradiography of rabbit parietal cell labeled with [^{3}H]omeprazole. (A) A representative electron micrograph of a parietal cell stimulated with 1 mM dbcAMP for 10 min in the presence of [^{3}H]omeprazole. The black arrows point to representative silver grains located over the canalicular compartment. The white arrow points to silver grains over the cytoplasmic compartment. (B) A representative electron micrograph of an unstimulated parietal cell at 10 min in the presence of [^{3}H]omeprazole.

pump required insertion into the canalicular membrane, then no significant increase in the number of grains would appear initially in the cytoplasmic compartment, but a large increase in the number of grains would be seen over the canalicular compartment.

In the data presented here, the results show that the stimulus dependent increase in the number of grains occurred over the canalicular compartment but not over the cytoplasmic compartment at the earlier time points where stimulation was occurring, in particular at 5 and 10 min. Hence, activation of the pump occurs upon or subsequent to insertion into the canalicular membrane. Activation of the pump does not occur while in the vesicular state.

Resting purified gastric vesicles from hog possess neither a K^+ nor a Cl^- permeability [5]. In the stimulated state, the rabbit H^+/K^+-ATPase is found in canalicular derived membranes which possess a KCl pathway, that appears to be conductive [8] but it is not clear whether these conductances are separate or physically coupled [8]. There is no evidence that the H^+/K^+-ATPase itself, is inactivated in the resting state. Activation appears to be solely a result of alteration of K^+ access to the outside surface of the pump.

The autoradiographic studies presented above imply that an active KCl pathway is absent from the pump while the pump is present in the cytoplasmic, vesicular state. cAMP or $[Ca]_i$ are second messengers known to stimulate acid secretion, by yet undefined phosphorylation pathways. The canalicular localization of the onset of acid secretion shows that either the activating kinases or the transporter(s) themselves are separate from the pump until canalicular insertion. In electrophysiological studies in *Necturus*, the apical membrane of the resting oxynticopeptic cell has been shown to have Cl^- channels [19] and to generate a Cl^- current [20]. This may indicate the presence of one component, the Cl^- conductance constantly present in the apical membrane of this species. Thus far, such studies have not provided data relating to the site of activation of the presumed K^+ conductance. Certainly, the separation of activation from the cytoplasmic form of the pump would prevent formation of acid spaces within the cytoplasm with possible osmotic rupture of tubulovesicles.

The finding that there is an increase in counts over the cytoplasmic compartment at later time points is interpreted as evidence for recycling of the pump. It cannot be accounted for by back diffusion of activated omeprazole since only the pump protein is labeled in the intact stomach [12]. Additionally, back diffused cationic sulfenamide would either break down very rapidly at relatively neutral pH to inactive molecules or react with the glutathione present in the cytoplasm, and be removed during preparation for autoradiography. Hence, the resting and stimulated configurations are not static, but equilibrium situations. In the unstimulated state, there is some cycling of the pump into the canalicular membrane with the equilibrium favoring the cytoplasmic pool of tubulo-vesicles. In the stimulated state there is a shift in the equilibrium that favors the canalicular location of the pump. This concept of continuous membrane recycling would account for the grains seen in the cytoplasmic compartment of parietal cells derived from either unstimulated or stimulated gastric glands and would account for the larger increase in counts over the cytoplasm at 30 or 60 min in cells derived from either stimulated or unstimulated glands. Stimulation could then be visualized as a shift of the equilibrium, where more pumps would be present at a given time in the canalicular membrane, with a resultant increase in secretion from basal to stimulated levels.

It is of interest that the appearance of counts over the cytoplasmic space appeared to be independent of the state of stimulation of the cell. Since these counts reflect recycling of the pump from the canalicular membrane back to cytoplasmic tubulo-vesicles, the retrieval pathway may not be influenced by the secretagogue cAMP. The balance between the cytoplasmic and canalicular location of the pump is affected by stimulation of the insertion pathway by an increase of the rate of insertion of the pump. During stimulation, retrieval of the pump is apparently not affected.

Acknowledgement

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