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Original articles

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FUNCTIONAL AND STRUCTURAL CHANGES OF ISOLATED RAT PARIETAL CELLS DURING MEMBRANE POTENTIAL MODULATION

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The present experiments were undertaken to extent our earlier observations (J Physiol Pharmacol 1991, 42, 367—79) relating membrane potential with membrane recycling of parietal cells. Studies were performed *in vitro* using gastric glands that were isolated through the use of rat stomachs transformed into "everted sacs" and filled with hyperosmolar NaCl-EDTA solution. Acid production was indirectly determined by accumulation of ^{14}C-aminopyrine (AP) and its translocation by measurement of acridine orange fluorescence. H^+/K^+-ATPase activity was assayed by measurement of K^+-stimulated p-nitrophenylphosphatase (pNPPase) of the proton pump. Morphologic state of parietal cells in relation to their functional activity was observed using electron microscopy. Changes in the membrane potential were obtained by the treatment of gastric glands with protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) in the incubation media of different pH. CCCP caused time-dependent decrease in AP accumulation by parietal cells from the medium of pH 6.6 but not that of pH 7.8. pNPPase activity increased in aplical and decreased in tubulovesical membrances prepared from CCCP treated glands which were incubated in the medium being more acidic than cell cytoplasm. Electron microscopic assessment showed morphological transformation of resting parietal cells treated with CCCP in pH 6.6 from nonsecreting to secreting state. CCCP acting in acidic incubation medium also caused the decrease in acridine orange fluorescence in the cytoplasm of parietal cells with some temporary increase of its fluorescence in the lumen o gastric glands.
These findings support our hypothesis that changes in parietal cell membrane potential by protonophore CCCP may translocate HCl from tubulovesicles to secretory canaliculi. While the above explanation is suggestive, the exact mechanisms controling a membrane recycling during the secretory response of parietal cells *in vitro* remain to be elucidated.

Key words: *gastric glands*, *aminopyrine accumulation*, *protonophore CCCP*, *membrane potential*, proton pump, acridine orange, *electron microscopy*

INTRODUCTION

Gastric acid secretion depends on two sequentional steps, i.e. the formation of acid and its translocation (1—3). In resting perietal cells tubulovesicles, which contain the activity of proton pump, do not communicate with the

canalicular and apical surfaces of the cell. Upon activation tubulovesicles incorporate into the apical cell membrane forming intracellular secretory canaliculi. After finishing the acid secretory process, tubulovesicles are formed from the apical membrane (4—6). Our previously published results (7) indicated that parietal cell membrane recycling may be dependent on changes in apical membrane potential. It seems that in chief cells, the second phase of pepsinogen secretion also depends on membrane potential (8).

The aim of this study was to determine the relationship between changes of the membrane potential caused by protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) and the distribution of H^+/K^+-ATPase actitity and structural findings in parietal cells of isolated rat gastric glands.

MATERIAL AND METHODS

Gland preparation

The gastric glands were isolated according to the method of Gespach et al. (9) through the use od the rat stomachs transformed into "everted sacs" filled with hyperosmolar NaCl-EDTA solution. After isolation glands were harvested by centrifugation, washed and resuspended in the incubation medium containing (in mM); 0.5 NaH_2PO_4, 1.0 Na_2HPO_4, 20 $NaHCO_3$, 70 NaCl, 11 glucose, 1.0 $CaCl_2$, 1.5 $MgCl_2$, 50 HEPES-NaOH, pH = 7.4, and 1 mg bovine serum albumine/ml. The cell viability of isolated glands, determined by exclusion of 0.4% trypan blue, was over 85% in used preparation.

Aminopyrine accumulation

^{14}C-aminopyrine (AP) accumulation was used as an index of acid secretory activity (10) and it was measured as described in detail previously (11). The gastric glands, liberated from the mucosa and washed in the incubation medium were divided into suitable portion and resuspended in the appropriate media. These media were the same as standard incubation medium except that each had a different pH. Media of the pH 6.6 and 7.8 were used. Replaced media containing 0.1 mM 3-isobutyl-1-methyl-xanthine (IBMX), 1 mM DTT, and 0.05 μCi ^{14}C-AP/ml were previously gassed for 5 min. with 95%O_2—5%CO_2. Glands were incubated in 1 ml medium without or with 10^{-4} M histamine for 45 min. at 37°C. Thereafter 10^{-6} M carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) was added and glands were incubated for 5, 10, and 15 min. more at 37°C.

All experiments were performed in triplicate. The ^{14}C-AP accumulation was calculated as an accumulation rato of ^{14}C-AP radioactivity in the parietal cells to that in the medium (10).

Assay of K^+-stimulated pNPPase activity of H^+/K^+-ATPase

Glands were preincubated for 45 min. in the incubation medium, pH 6.6 or 7.8 containing 1 mM IBMX and 1 mM DTT without or with 10^{-4} M histamine. Thereafter glands were incubated for 10 min. more at 37°C with or without 10^{-6} M CCCP. Then glands were harvested by centrifugation, washed twice in ice-cold PBS and homogenized in 5 ml of ice-cold homogenizing medium containing (in mM): 125 mannitol, 40 sucrose, 1 EDTA, and 5 PIPES/Tris buffer,

pH = 6.7 (12), using a Potter-Elvenhjem (teflon-glass) homogenizer. The homogenate was centrifuged at 40 g_{max} for 10 min. The supernatant was centrifuged at 4300 g_{max} for 10 min. The pellet from the 4300 g spin (P_1) was retained and the supernatant recentrifuged at 14600 g_{max} for 10 min. The supernatant from the 14600 g spin was recentrifuged at 50000 g_{max} for 90 min., and the pellet from this spin was retained. Pellets were resuspended in the medium containing (in mM): 300 sucrose, 0.2 EDTA, and 5 Tris-HCl buffer, pH = 7.2.

p-nitrophenylphosphatase (pNPPase) activity was measured in 0.5 ml of the medium containing 30 μg of protein, 5 mM $MgSO_4$, 5 mM Na_2pNPP, 100 μM ouabain, 10 mM PIPES/Tris buffer, pH = 7.2, either with or without 10 mM KCl (12). Incubations were carried out at 37°C for 10 min. and the reaction was stopped by addition of 0.75 ml of 0.1 M NaOH. After protein precipitation, the optical density of p-nitrophenolate was read at 410 nm. All enzymatic determinations were performed in triplicate.

The protein concentration was measured using Micro BCA protein assay, Pierce Chemical.

Acridine orange uptake

Gastric glands were preincubated for 30 min. at 37°C in the incubation media, pH 6.6 and 7.8 with 10^{-4} M histamine. Glands suspensions loaded with 100 μM acridine organge for 10 min. at 37°C were thereafter incubated without or with 10^{-6}M CCCP and fluoresence was monitored within 10 minutes in Olympus IMT-2 inverted microscope, equipped with vertical fluorescence illuminator. Serial color pictures were taken from each incubation.

Electron microscopic studies

For ultrastructural examinations, the gastric glands incubated without or with 10^{-4}M histamine in the incubation media pH 6.6 and 7.8 and thereafter treated with 10^{-6} M CCCP for 10 min. or untreated were fixed in 2% solution of glutaraldehyde buffered with 0.2 M cacodyl buffer, pH = 7.4, routinely processed, and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate. All observations were carried out using JEM 100C electron microscope, equipped with a gonometric stage at magnifications of $\times 4000 - \times 20\,000$.

Data are expressed as means $\pm$SD. Significance was determined by the Student's test.

RESULTS

Preliminary experiments revealed that a steady-state accumulation of ^{14}C-AP is achieved during 45 min. of exposure of gastric glands to histamine. The response was concentration-dependent and the maximal effect was achieved at 10^{-4} M histamine. In all experiments, this saturating concentration of hitamine was used plus 10^{-4} M IBMX to inhibit phosphodiesterase. At saturating concentrations, ^{14}C-AP accumulation ratios increased from 10.4 ± 3.5 (n = 13) to 96 ± 21 (n = 13) with histamine stimulation. At the concentration used, IBMX alone had no effect on basal AP accumulation.

As shown, 10^{-6}M CCCP affected basal (*Fig. 1*) and histamine-stimulated (*Fig. 2*) AP accumulation by gastric glands resuspended in the incubation medium pH = 6.6, but not in the medium pH = 7.8. The inhibitory effect of CCCP increased progressively at increasing time of the incubation. At pH 7.8

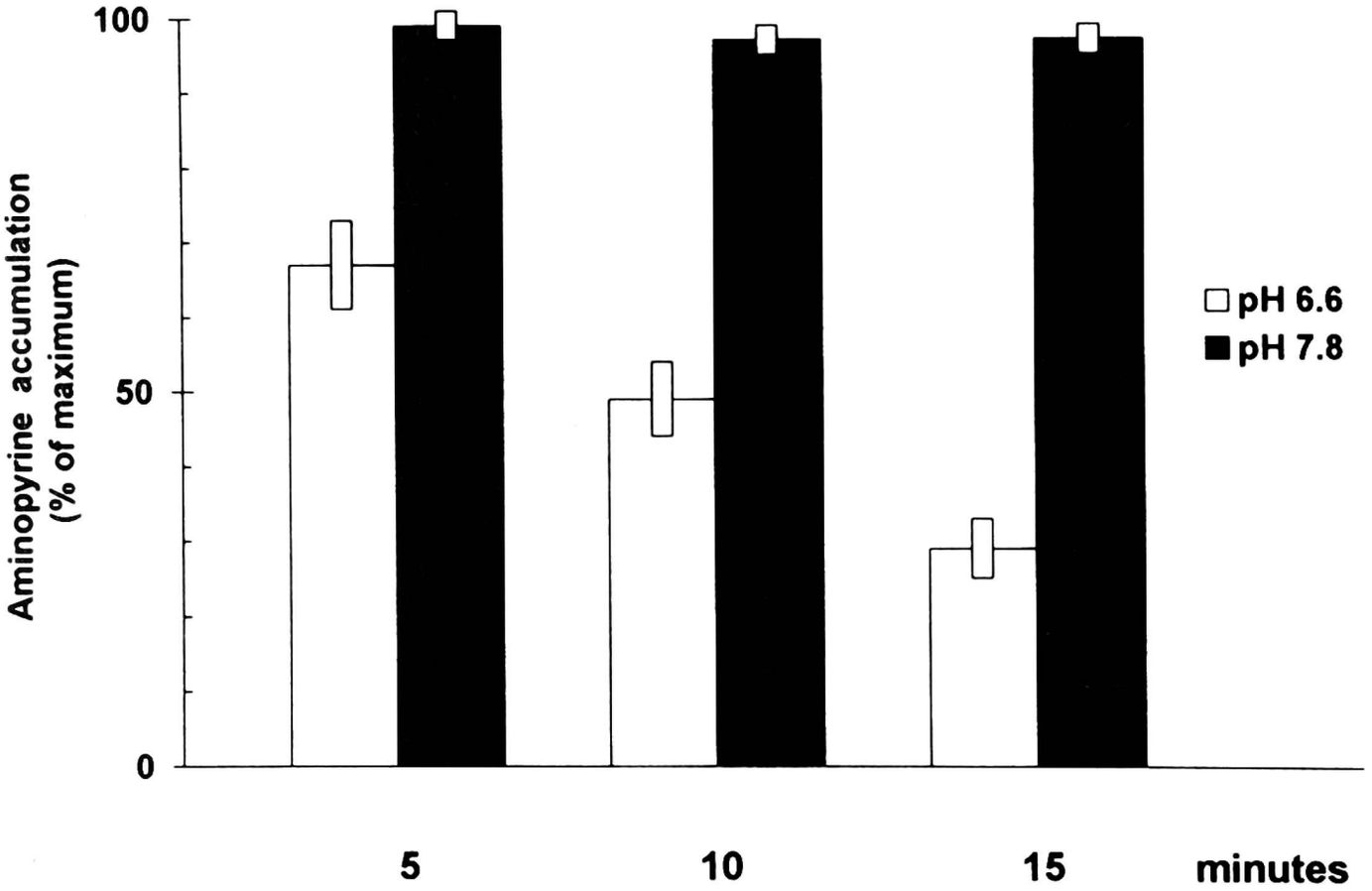

Fig. 1. Graph showing the inhibitory effect of 10^{-6} M CCCP on basal aminopyrine accumulation as a function of pH of the incubation medium. Results are expressed as percentage inhibition of basal aminopyrine accumulation effected by CCCP treatment. In this experiments basal aminopyrine accumulation averaged 9.7 ± 2.1. Results represent means $\pm$ SD of 5 experiments performed on different cell preparations.

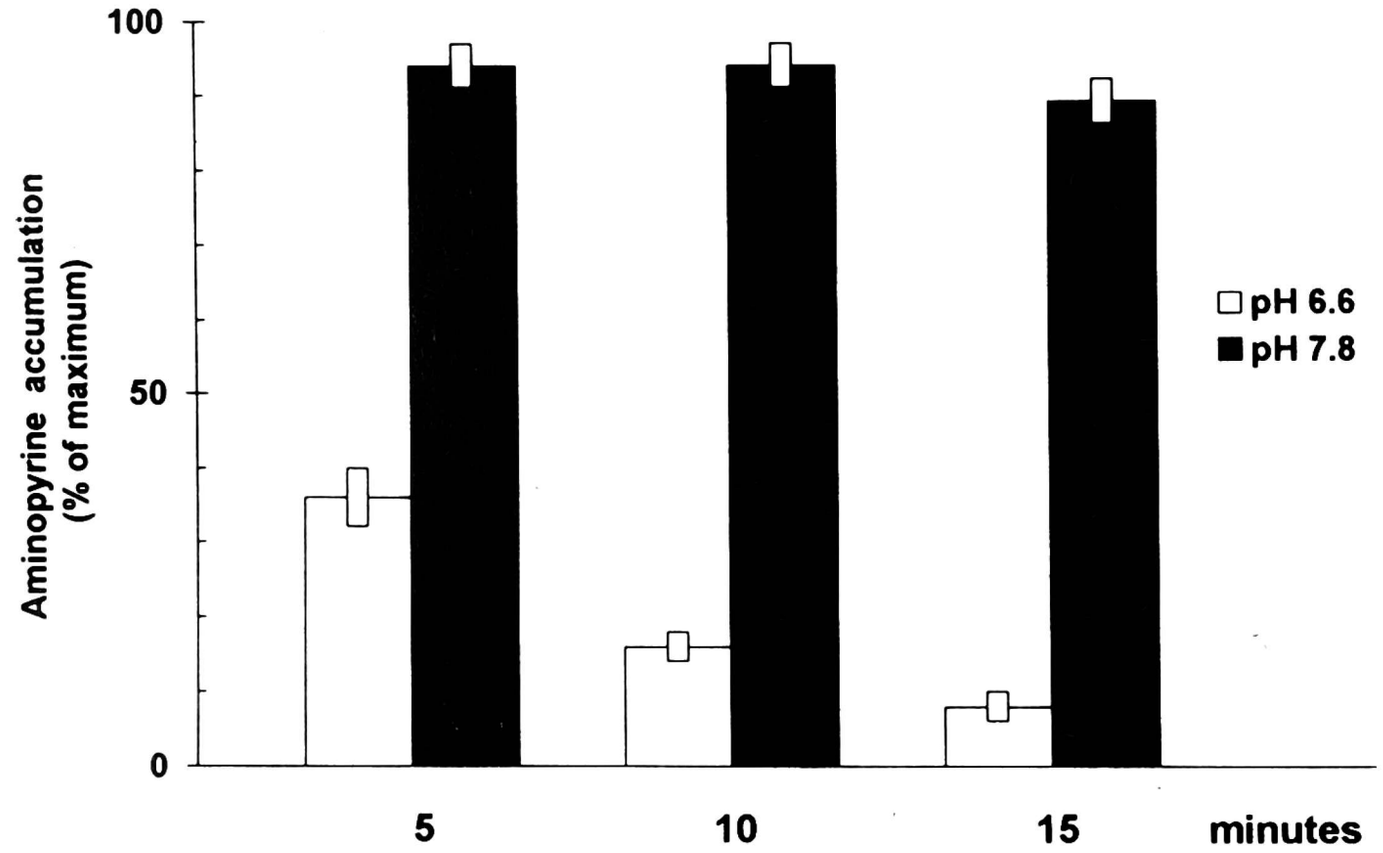

Fig. 2. Graph showing the inhibitory effect of 10^{-6} M CCCP on histamine-stimulated aminopyrine accumulation as a function of pH of the incubation medium. Results are expressed as percentage inhibition of histamine-stimulated aminopyrine accumulation effected by CCCP treatment. In this experiments basal aminopyrine accumulation averaged 86.3 ± 16.8. Results represent means $\pm$ SD of 5 experiments performed on different cell preparations.

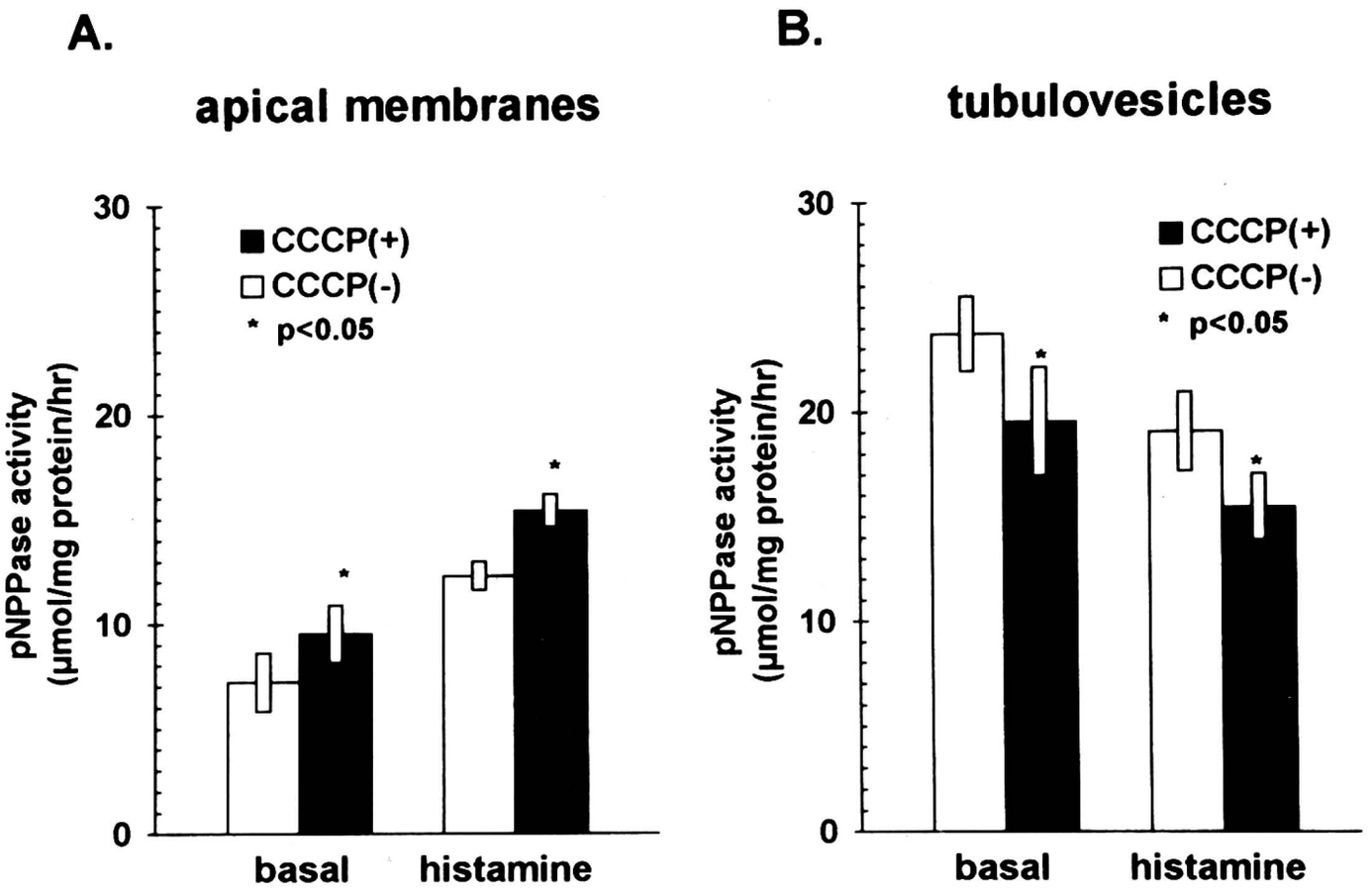

Fig. 3. Graph showing effects of CCCP treatment on the distribution of specific activity of K^+-stimulated p-nitrophenylphosphatase (pNPPase) among apical membranes (Graph A) and tubulovesical membranes (Graph B) prepared from resting and histamine-stimulated gastric glands which were incubated in the medium of pH 6.6. Results represent means ± SD of 4 experiments performed on different cell preparations. (*) indicates significant ($p < 0.05$) increase or decrease of K^+-pNPPase activity of CCCP-treated glands incubated in the medium of pH 6.6 compared to those untreated.

of the incubation medium, CCCP had significant effect on histamine--stimulated AP accumulation only after 15 min. of the ionophore treatment.

Since many problems complicate the use of the assay of hydrolysis of ATP by H^+/K^+-ATPase, particularly when its activity can be affected by membrane potentials, in this study the K^+-stimulated pNPPase activity of proton pump was assayed. Specific activities of pNPPase in the apical membrane P_1 and tubulovesicles P_2 isolated from unstimulated and histamine-stimulated gastric glands incubated in the medium of pH 6.6 are presented in *Fig. 3*. The pNPPase activities in the apical membranes of unstimulated glands were lower than those of histamine-stimulated glands. In contrast, the pNPPase activities of tubulovesicles were lower in histamine-stimulated than in unstimulated glands.

There ware no significant differences of the enzyme activities between preparations obtained in gastric glands incubated in the medium pf pH 6.6 and that of 7.8 (data not shown).

For gastric glands incubated in the medium of pH 6.6, pNPPase activity was increased by CCCP treatment in both unstimulated and histamine-stimulated

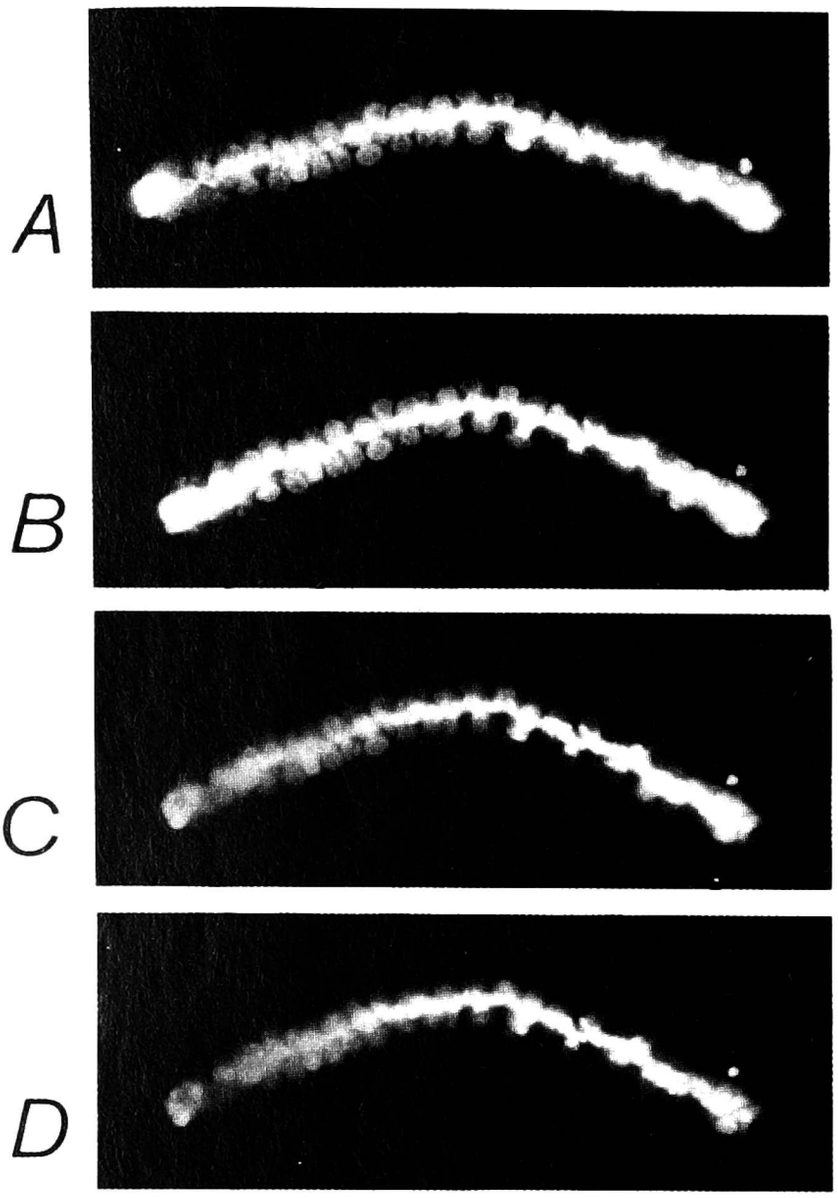

Fig. 4. Series of fluorescent photomicrographs: Histamine-stimulated gastric gland incubated in the medium of pH 6.6 in the presence of 100 μM acridine orange (A); Stimulated gastric gland as in (A): 1 minute (B), 3 minutes (C), and 6 minutes (D) following addition of 10^{-6} M carbonylcyanide m-chlorophenylhydrazone (CCCP). The loss of red fluorescence from glandular cells is seen.

apical membranes P_1 to a mean of 132 ± 5 and $125 \pm 2\%$, respectively. In tubulovesicles P_2, CCCP treatment had some inhibitory effect on pNPPase, decreasing its activity to $82 \pm 6\%$ compared to unstimulated and to $89 \pm 2\%$ compared to histamine-stimulated gland enzyme activity. For membrane preparations from glands incubated in the medium of pH 7.8, CCCP treatment did not change the specific activity of the enzyme in both P_1 and P_2 membrane preparations (not shown).

Examination by fluorescence microscopy of unstimulated gastric glands loaded with acridine orange showed green fluorescence corresponding to 510 nm emission peak (13). In histamine stimulated glands, red fluorescence spread over a large part of the cytoplasm of triangular, peripherally located parietal cells was noted (*Fig. 4*). The distribution of red fluorescence differed between parietal cells in the glands as well as between glands. In some cells, well organized structure of canaliculi system could be observed, in other the cytoplasmic red fluorescence was much weaker or was not visualized. In stimulated glands also some red fluorescences was seen in the lumen of the glands. Treatment of stimulated gastric glands with CCCP caused

decrease in red fluorescence intensity located in parietal cell cytoplasm with some temporary increased fluorescence of accumulated acridine orange in the gland lumen.

For ultrastructural studies, microphotographes of sections from each incubation condition of 4 experiments performed on different gland preparations, randomly choosen, were analysed. Parietal cells were observed in a variety of different morphologic states which varied with functional activity. In untreated gastric glands, most of parietal cells were morphologically nonsecreting, i.e. they typically contained prominent intracytoplasmic smooth surface membranes, termed as the tubulovesicular system, numerous dense mitochondria, and a round nucleus. Following histamine stimulation, the tubulovesicles decreased in number, and the intracellular canalicular system was in an open state, i.e. large diameter intracellular secretory canaliculi with the microvilli were observed.

Parietal cells from resting gastric glands, treated with 10^{-6} M CCCP for 10 minutes, also lost their intracytoplasmic system of tubulovesicles with its transformation into large expended canaliculi without microvilli. Therefore, microphotographs of these CCCP treated cells showed pictures similar to histamine stimulated parietal cells. Histamine stimulated parietal cells treated with CCCP revealed large mitochondria, typically found in active secretion state, with the presence of canaliculi and vacuoles.

DISCUSSION

Initiation of gastric acid production is mediated by specific receptors on the surface of parietal cells (11). Histaminergic H_2, cholinergic, and gastrinergic receptors activate two separate cAMP- and Ca^{+2}-dependent pathways enhancing pumping of protons by the K^+/H^+-ATPase. The resting parietal cell is characterized by an apical membrane with short microvill and prominent intracytoplasmic tubulovesicular system. Activation of parietal cell by secretagogues results in decrease in number of tubulovesicles and increase in the apical membrane surface. Upon activation a proton pump translocation into the apical membrabe and increased cellular oxygen uptake are observed. The mechanism of the membrane recycling in parietal cell is not clear.

The present experiments were undertaken to extent the results of our earlier study which could confirm the speculative hypothesis relating membrane recycling with membrane potential (7).

The protonophore CCCP transports H^+ ion towards the more basic side of the cell membrane if the pH difference between the cytoplasm and the membrane exists (14—16). Assuming resting and stimulating cytoplasmic pH as 6.97 and 7.1, respectively, CCCP treated parietal cells in the medium of

pH = 6.6 reach membrane potential +22 mV in resting and +30 mV in stimulated cells, and in the medium of pH = 7.8, −50 mV and −42 mV, respectively (17).

CCCP caused time-dependent decrease in the AP accumulation by parietal cells from the medium being more acidic than cytoplasm, whereas cells incubated in the medium being basic than cytoplasm did not change their AP accumulation after CCCP treatment. As predicted by our hypothesis, this decrease in AP accumulation results from the incorporation of tubulovesicles into the cell membrane and the release of their content into the medium. In fact, CCCP treatment caused changes in the distribution of the proton pump between fractions of apical and tubulovesical membranes. In both resting and histamine-stimulated gastric glands treated with the protonophore, K^+-stimulated pNPPase activity of H^+/K^+-ATPase increased in apical membrances being decreased in those of tubulovesical in the preparations from glands incubated in the medium of pH 6.6, but not in the medium of pH 7.8.

In ultrastructural studies, resting parietal cells were mostly morphologically nonsecreting. Histamine-stimulated cells showed changes being considered as those morphologically secreting. Their intracellular canalicular system was observed in an open state, i.e. microphotographes revealed secretory canaliculi with loss of tubulovesicles. Unstimulated parietal cells treated with CCCP in the incubation medium of pH 6.6, but not in the medium of pH 6.6, but not in the medium of pH 7.8, were not more representative of the resting cells. Similar to histamine-stimulated cells, large expansion of the intracellular canaliculi concomitant with the absence of the cytoplasmic tubulovesicles were found. Thus, electron microscopic assessment after treatment of gastric glands with CCCP in more acidic incubation medium showed morphological transformation of parietal cells.

Acridine orange has been used to examine pH gradients in vesicular systems (13, 18). This dye binds to negatively charged molecules, i.e. DNA. Depending on dye concentration, a green color fluorescence with a peak at 510 nm is visualized at its lower concentrations, and a red fluorescence with maximum emission at 600 nm with higher dye concentrations appears (13). Acridine orange accumulation indicates high proton concentration in acid-secreting parietal cells and it can be used as a probe of pH gradient. Observations of fluorescence intensity changes after parietal cells loading with acridine orange could not be used in this study as quantitative tool of acid distribution among gastric glands since measurement of the signal by a fluorescence detector was not available. However, even in spite of these limitations, it seems that the obtained results are the additional evidence strengthening hypothesis that decreased AP accumulation by parietal cells in the presence of CCCP may depend on acid secretion from parietal cell secretory system.

In summary, our results showed that decreased AP accumulation resulted from the induction of positive membrane potential by the use of CCCP treatment in acidic medium was accompanied with the translocation of the proton pump activity from tubulovesical to apical membranes and the expansion of intracellular canaliculi as assessed by microscopic studies. These findings support, therefore, the hypothesis that the decrease in AP accumulation due to changes in the membrane potential may be a consequence of acid release from parietal cells to the medium. While the above explanation is suggestive, the exact mechanisms controling a membrane recycling during the secretory response of parietal cells *in vivo* remain to be elucidated.

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