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DOES THE MEMBRANE POTENTIAL CONTROL INCORPORATION OF TUBULOVESICLES INTO THE SECRETING APICAL MEMBRANE OF THE RAT PARIETAL CELL?

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The present studies were designed to examine the effect of changes in membrane potential by means of protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) and variations in the pH of the medium on the secretory response of parietal cells. Studies were performed in vitro using isolated cells from rat stomachs and acid production was indirectly determined by ^{14}C-aminopyrine (AP) accumulation. CCCP affected both basal and histamine-stimulated AP accumulation in a concentration-dependent manner. The AP accumulation ratios depended on pH of the incubation medium; the ratio was lowest at pH 6.6, and increased progressively as the pH of the medium increased to 7.8. Moreover, the decreases in AP accumulation ratios caused by simultanous addition of CCCP and AP to cell suspensions compared to those in which CCCP was added to incubated cells after achieving the steady-state of AP accumulation were quantitatively similar. These findings suggest that the decrease in AP accumulation due to CCCP treatment is a consequence of an activation of acid secretion rather than an inhibitor of acid production. From the present and previously published data, we propose a working hypothesis: membrane recycling is dependent on changes in apical membrane potential.

Key words: rat parietal cells; aminopyrine accumulation; membrane potential; protonophore

INTRODUCTION

The resting parietal cell is rich in tubulovesicles which upon activation are incorporated into the apical cell membrane forming intracellular secretory canaliculus. When the secretory process is finished tubulovesicles are formed from the apical membrane (1—3). The mechanism controlling such a membrane recycling process is unknown. It has been suggested (4) that the membrane potential plays the key role i controlling membrane recycling in other systems.

In this study we tried to determine whether changing the mebrane potential by means of the protonophore carbonyl cyanide m-chlorophenylhydrazone and variations in the pH of the medium can mimic the secretory response of isolated rat parietal cells.

MATERIAL AND METHODS

Reagents: [Dimethylamine-^{14}C]-aminopyrine was obtained from New England Nuclear Research Products, and all other chemicals were from Sigma (St. Louis, Mo.).

Cells. Usually, two or three nonfasted female Wistar rats, weighing 240—300 g. were used per experiment. The isolation of cells was performed according to the method of Gespach et al (5) through the use of the stomachs transformed into "everted sacs". Each sac was filled with 3 ml of ice-cold solution containing 2.5 mM EDTA in 0.25 M NaCl, pH = 7.5. The sacs were incubated in the same solution on ice for 30 min. Thereafter, the gastric glands were liberated from the mucosa by hand shaking for 10 sec. After shaking, isolated glands were harvested by centrifugation (200 × g for 2 min), washed and resuspended three times in the incubation medium containing (in mM): 0.5 NaH_2PO_4, 1 Na_2HPO_4, 20 $NaHCO_3$, 70 NaCl, 5 KCl, 11 glucose, 1 $CaCl_2$, 1.5 $MgCl_2$, 50 HEPES--NaOH, pH 7.4, and 1.0 mg bovine serum albumin/ml, and stomachs were shaken again in 25 ml of fresh isolating medium. This procedure was repeated several times until no more glands were obtained.

The gastric glands were further mechanically dispersed into single cells by repeated aspiration into a Pasteur pipette with a fire-polished tip. The dissociated product, consisting of single cells and small clumps was then filtered through a 60 μm nylon mesh. Isolated cells were collected by centrifugation at 200 × g for 5 min. and finally suspended in the same incubation medium. The viability of isolated cells, determined by exclusion of 0.4% trypan blue, was over 90% in all preparations. Each cell separation yielded 18—28% of parietal cells, identified by examination with a light microscope.

^{14}C-*aminopyrine accumulation* was used as an index of acid secretory activity. Accumulation of ^{14}C-aminopyrine was measured as described in detail previously (6). Briefly, after 10 minute preincubation at 37°C in incubation medium containing 0.1 mM 3-isobutyl-1-methyl-xanthine (IBMX) gassed with 95% O_2—5% CO_2, crude cell suspension was divided into suitable portions, then the cells were harvested by centrifugation (200 × g for 5 min.) and resuspended in the appropriate media. These media were the same as the standard incubation medium except that each had a different pH. Media of the following pH values were used: 6.6, 7.0, 7.4, and 7.8. Replaced media containing 0.1 mM IBMX and 0.05 μCi ^{14}C-aminopyrine per each 5 × 10⁶ cells were previously gassed for 5 min. with 95% O_2—5% CO_2. Next 5 × 10⁶ cells were incubated in 1 ml medium without or with 10⁻⁴ M histamine and 10⁻⁶ or 10⁻⁷ M carbonylcyanide m-chlorophenylhydrazone (CCCP) in capped 1.5 ml Eppendorf tubes for 60 minutes more at 37°C. In some experiments, 5 × 10⁶ cells/ml were incubated in appropriate media previously gassed with 95% O_2—5% CO_2, containing 0.1 mM IBMX and 0.05 μCi $_{22}$C-aminopyrine without or with histamine (basal and stimulated AP accumulation, respectively) for 45 minutes at 37°C. Thereafter, the protonophore CCCP was added and cells were incubated for 15 minutes more at 37°C.

All experiments were performed in triplicate. The ^{14}C-aminopyrine accumulation was calculated as an accumulation ratio of ^{14}C-aminopyrine radioactivity in the parietal cell to that in the medium (6).

Data are expressed as means ±SD. Significance was determined by Student's test.

RESULTS

Cell membrane potentials can be changed by gradients of H+ in the presence of protonophores which passively transport protons down an electrochemical gradient, producing a membrane potential equal to the Nernst potential for protons (7—9). In this study we determined, therefore, the effect of the weak acid protonophore, carbonylcyanide m-chlorophenylhydrazone (CCCP) on AP accumulation in standard medium at four different level of pH: 6.6, 7.0, 7.4, and 7.8.
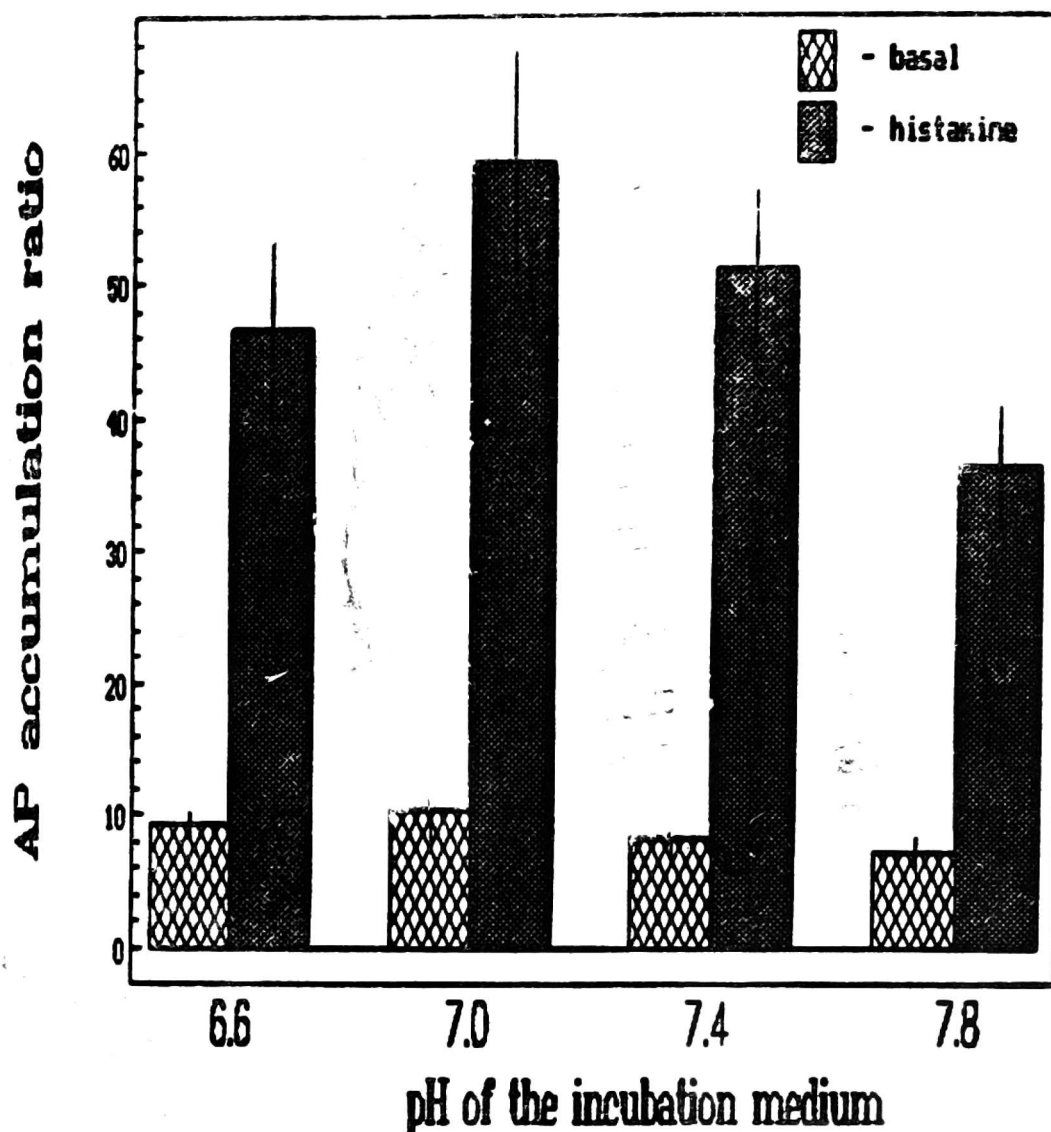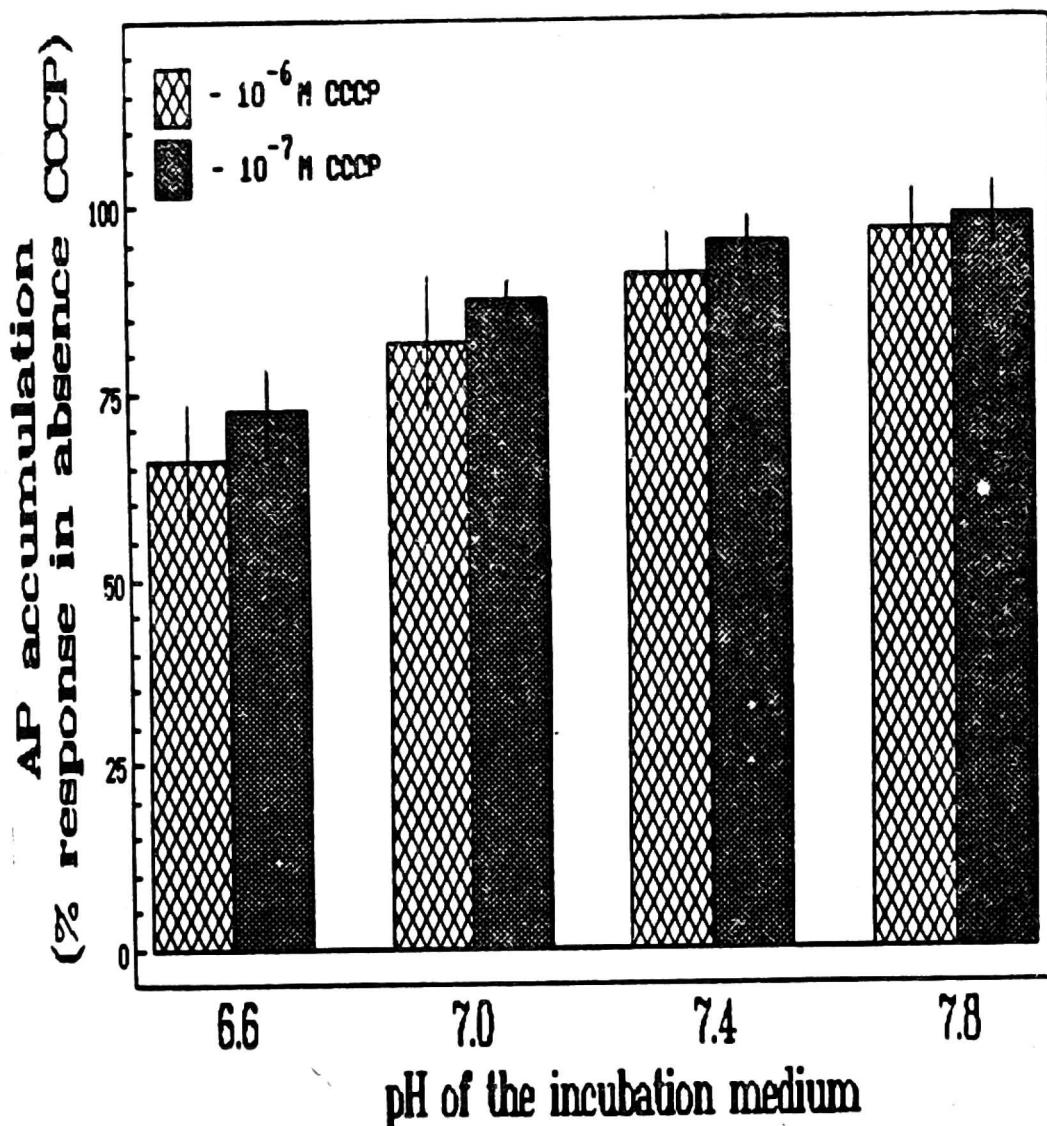

Fig. 1. Effect of the pH of the incubation medium on basal and histamine-stimulated aminopyrine (AP) accumulation. Results represent mean ±SD of four experiments.

Crude cell fractions were used in all experiments on acid production by indirect measurement with AP accumulation. Preliminary experiments showed that 45 min exposure of parietal cells to histamine was required to achieve a steady-state accumulation of ^{14}C-aminopyrine. The response was concentration-dependent and the maximum effect was observed at 10^{-4} M histamine.

10^{-4} M IBMX was used to inhibit phosphodiesterase. At this concentration IBMX alone had no significant effect on basal AP accumulation.

Fig. 1 presents results of the measurement of the effect of changes in pH in the standard incubation medium on the basal and histamine-stimulated AP accumulation. Effects of medium pH on AP accumulation were not significant with the exception of histamine-stimulated AP accumulation ratios found at pH 7.8 which were significantly lower ($p > 0.05$) than those found at pH 7.0 and 7.4.

As shown, CCCP affected the basal (*Fig. 2*) and histamine-stimulated AP accumulation (*Fig. 3*) in a concentration-dependent manner. The AP accumulation ratios depended on pH of the incubation medium; they were lowest at the lowest pH, and increased progressively at increasing pH of the medium. These inhibitory effects of CCCP at lower pH were, however, much more striking for the histamine-stimulated than for the basal AP accumulation.

Fig. 2. Inhibitory effects of CCCP (10^{-6} and 10^{-7} M) on basal aminopyrine (AP) accumulation as a function of pH of the incubation medium. Results are expressed as the percentage of unstimulated AP accumulation observed in absence of CCCP at varying pH as indicated. Results represent mean ±SD of four experiments.

In the other experiments, resting and histamine stimulated parietal cells were preloaded with AP in media of different pH values to achieve a steady-state of AP accumulation, and then, they were incubated in the presence of CCCP for 15 minutes more (*Fig. 4* and *5*).
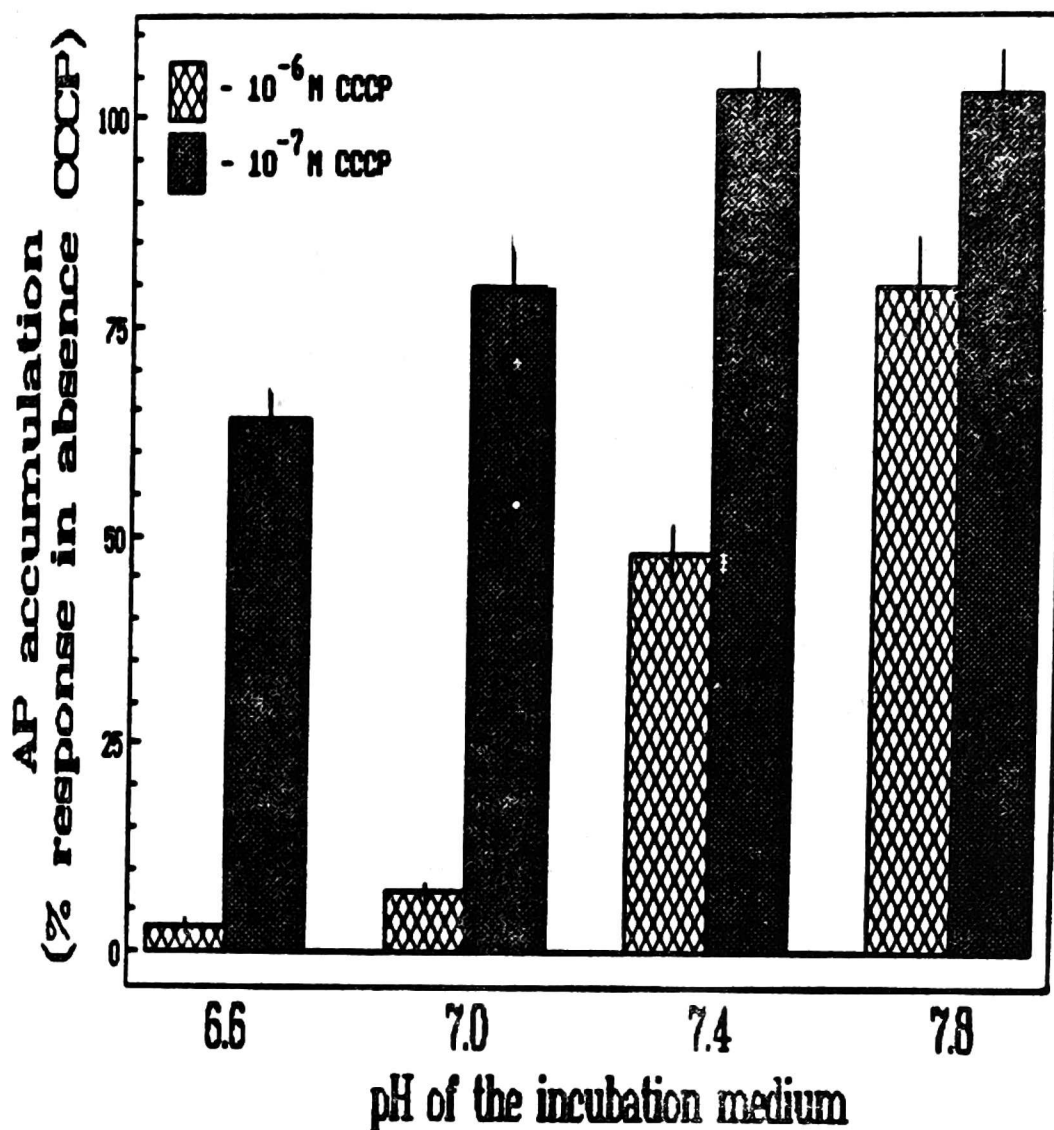

Fig. 3. Inhibitory effects of CCP (10^{-6} and 10^{-7} M) on stimulated aminopyrine (AP) accumulation as a function of the pH of the incubation medium. Results are expressed as percentage of stimulated AP accumulation observed in absence of CCCP at varying pH as indicated. Each value of stimulated AP accumulation was calculated as the difference between histamine-stimulated and basal AP accumulation ratio for each specified pH. Results represent mean $\pm$SD of four experiments.

Thus, we did the experiment in two ways. First, CCCP and ^{14}C-aminopyrine were added simultaneously to parietal cell suspension. Second, the parietal cells were allowed to equilibrate their level of ^{14}C-aminopyrine, and then the CCCP was added. In both protocols, the decrease in AP accumulation caused by the CCCP was quantitative similar.
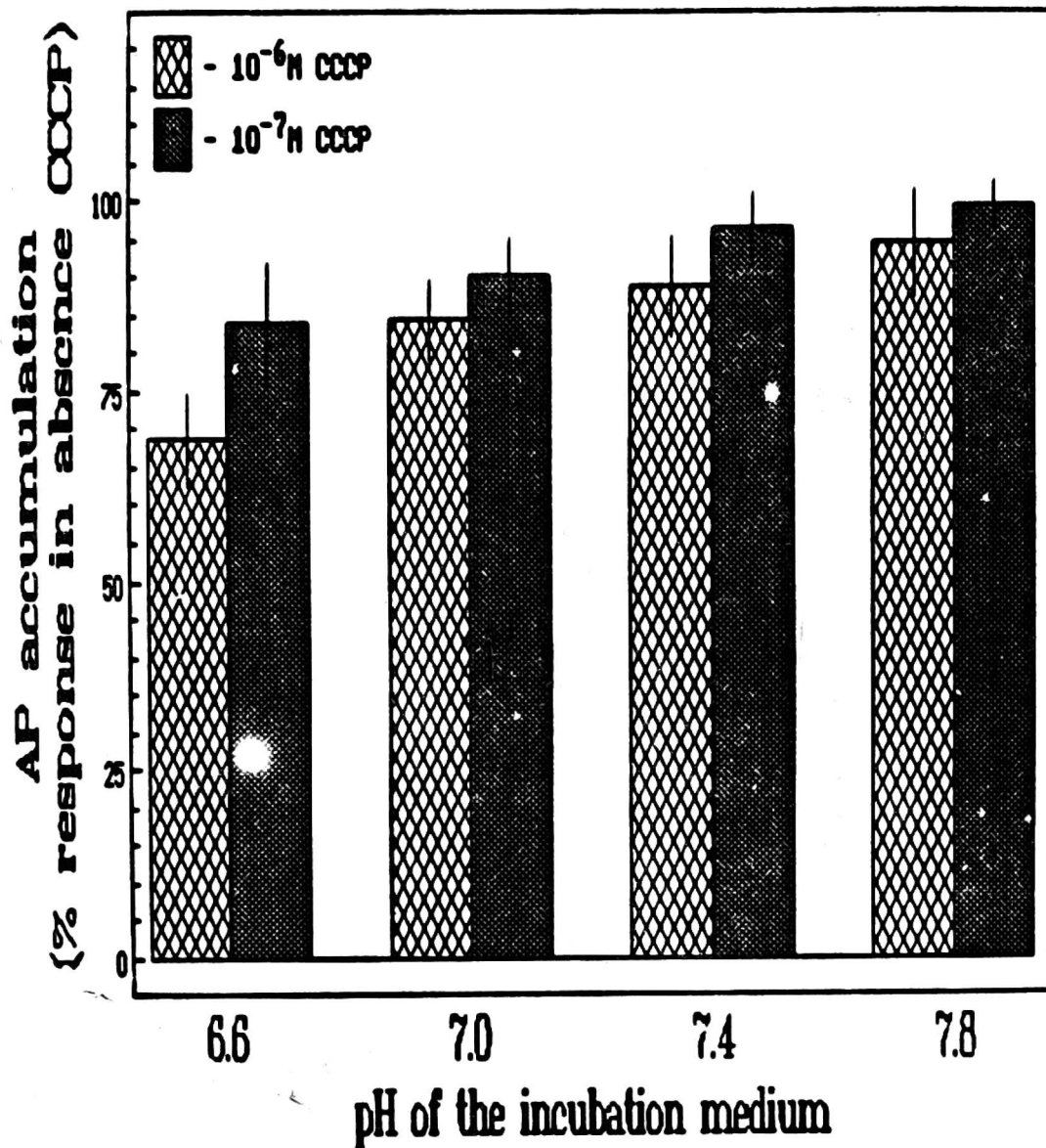
Fig.4. Medium pH-dependent decrease in basal steady state AP accumulation resulting from CCCP treatment. Parietal cells were incubated for 45 minutes in the presence of ^{14}C-aminopyrine in media of different pH: 6.6; 7.0; 7.4; 7.8, and then CCCP to final concentration of $10^{-6} — 10^{-7}$ M was added for the next 15 minutes of incubation. Results are expressed as percentage of unstimulated AP accumulation achieved after 60 minute incubation in absence of CCCP at varying pH as indicated. Results repersent mean ±SD of four experiments.

DISCUSSION

There are different ion pumps present in basolaternal and apical membranes of the parietal cell (10, 11). The ion transport mechanisms present in the apical membrane are similar to those in tubulovesicles. There is a positive potential inside the tubulovesicle (10) which inhibits K^+-H^+-ATP-ase activity (12). Upon activation of parietal cell the additional chloride transport route opens in tubulovesicle and in apical membranes and HCl is produced (10, 13). Subsequently, tubulovesicles are incorporated into apical membranes releasing their contents into the mucosal side of the gastric tissue (1, 2, 3). Although the cause of the membrane recycling is not clear, general consideration of
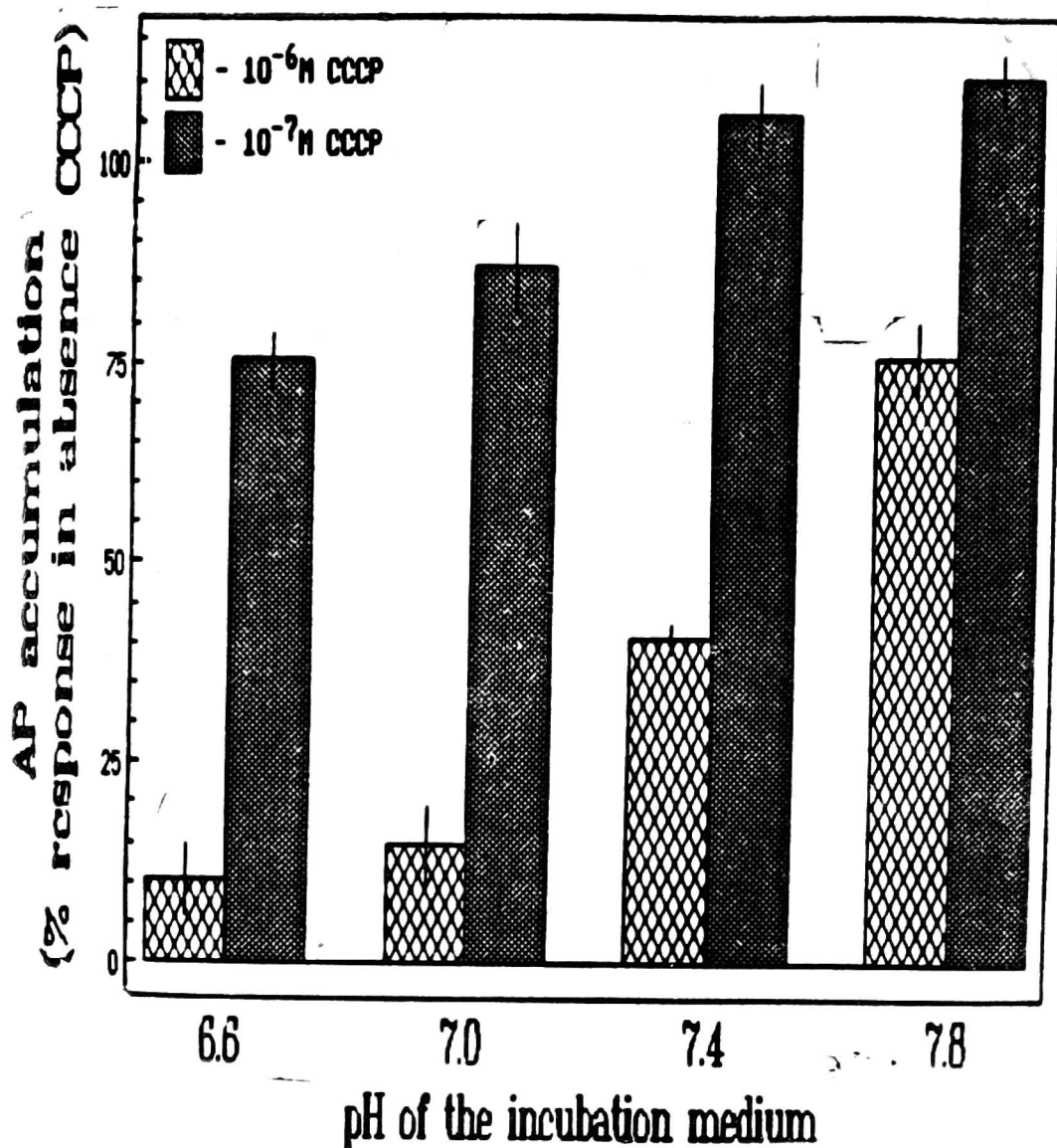
Fig. 5. Medium pH-dependent decrease in histamine-stimulated steady state AP accumulation resulted from CCCP treatment. Parietal cells were incubated for 45 minutes in the presence of ^{14}C-aminopyrine and histamine in media of different pH: 6.6; 7.0; 7.4; 7.8; and then CCCP to final concentration of 10^{-6} and 10^{-7} M was added for the next 15 minutes of incubation. Results are expressed as percentage of stimulated AP accumulation achieved after 60 minute incubation in absence of CCCP at varying pH as indicated. Each value of stimulated AP accumulation was calculated as the difference between histamine-stimulated and basal AP accumulation ratio for each specified pH. Results represent mean ±SD of four experiments.

the energetics of membrane recycling (4) suggests that membrane potential could play a role in such a process.

The isolation od parietal cell from the tissue causes the mixing of the ion pumps of basolateral and apical membranes. This phenomenon might obscure the directional processes present in intact tissue and short circuit potential differences between apical and basolateral surfaces.

Aminopyrine accumulation is considered to be a measure of secretory activity of parietal cells. In fact, aminopyrine is stored exclusively in tubulovesicles and upon association of these vesicles with the apical membrane and secretion of their contents, the AP accumulation ratio actually decreases.

This should be always kept in mind when analyzing the experiments with aminopyrine. The pH of secreted HCl by an activated cell is in the range of 0.8 to 1. Since the AP ratio increases 5 to 10 fold upon activation of parietal cell one could wrongly assue that the pH in resting tubulovesicles is 0.7 to 1 unit higher then in a resting one, while in fact, the pH of the resting tubulovesicle contents is in the range of 4 (10). However, one should take into account that the number of tubulovesicles decreases many times upon activation (3).

The CCCP molecule is a protonophore which has ability to transport H^+ ion down its electrochemical gradient (7—9). If there is a pH difference between the cytoplasm and the medium then protons are transported towards the more basic side of the cell membrane. The membrane potential value produced due to proton transport across the cell membrane is given by the Nernst equation. In the media of pH 6.6, 7.0, 7.4, 7.8 in the presence of CCCP the resting parietal cell membrane potential value reaches $+22$, -2, -26, and and -50 mV, respectively (assuming cytoplasmic pH $= 6.97$ (14)) and the stimulated parietal cell membrane potential value reaches $+30$, $+6$, -18, -42 mV, respectively (assuming cytoplasmic pH $= 7.1$ (14)).

Since in our experiments CCCP causes a dramatic decrease in the aminopyrine accumulation ratio mainly in the media which are more acidic then the cytoplasm, the effect observed cannot be caused by the action of CCCP molecules on cell organelles like mitochondria or tubulovesicles itself, because the effect would not dependent upon the pH of the medium. There are two possible explanations for the observed phenomenon. First, there might be a perturbation in the ion transport mechanisms due either to high proton membrane conductivity or the membrane potential change. The alternative explanation is based on the hypothesis relating membrane recycling with membrane potential (4). As predicted by this hypothesis, the decrease in AP ratio is caused by incorporation of tubulovesicles into the cell membrane and the release of their contents into the medium.

The energetic consideration (4) leads to the conclusion that the vesicle made of negatively charged lipid molecules could be incorporated to the cell membrane if membrane potential is negative and the potential across the vesicle membrane is negative. On the other hand, the vesicle could be formed from the cell membrane only in the areas of positive (or zero) membrane potential value. However, when analyzing the parietal cell one cannot safely assume that the membrane lipids are net negatively charged because the pH on the internal side of a tubulovesicle and luminal side of the apical membrane can reach the value of 1. In the neutral medium lipid molecules are either net negatively charged (phosphatidylserine, phosphatidylinositol, phosphatidic acid, diphosphatidylglycerol), zwitterionic (phosphatidylethanolamine,

phosphatidylcholine, sphingomyelin) or neutral (cerebroside, cholesterol). In very acidic medium the phosphate groups of phospholipids (negatively charged in neutral medium) become neutral (pK $= 2.1$) and the amide groups of cerebroside and sphingomyelin (non charged in neutral medium) become positive (pK $= 0.6$). Thus, at the pH lower then 3, lipids become net positively charged. At pH below 3, energetic considerations predict that vesicles will be incorporated into the cell membrane if the membrane potential is positive and the potential across the vesicle membrane is also positive. Contrare to the process of vesicle incorporation into plasma membrane, the vesicles could be formed from the cell membrane when the membrane potential is zero or negative. The previously published data confirm that the potential in tubuovesicles is positive (12). The results of our study indirectly indicate that when the membrane potential becomes positive, tubulovesicles are associated with the cell membrane of parietal cell.

From the already established facts: 1. membrane recycles upon activation from tubulovesicles to apical membrane and after secretion back to tubulovesicles (1—3); 2. the potential inside tubulovesicles is positive (10); 3. a positive potential inhibits K^+–H^+–ATP-ase activity (12); 4. the pH values inside the resting and activated vesicle are 4 and 1, respectively (10); 5. upon activation a Cl^- route opens (12, 13); 6. induction of secretion by applying a negative potential on the luminal side and a positive potential on the serosal side of the gastric mucosa (14—19); 7. our experiments suggesting tubulovesicle incorporation into the apical cell membrane after induction of positive membrane potential by the use of CCCP treatment in acidic media; 8. and the energetics of membrane recycling (4) — the following membrane recycling pattern in parietal cell might be deduced — *Fig. 6.*

In the resting state there are numerous tubulovesicles. The internal pH of these vesicles is 4, their lipids are negatively charged, their transmembrane potential is positive due to K^+ ion leakage from cytoplasm, and positive potential inhibits K^+–H^+–ATP-ase activity. In the resting state, the apical membrane potential is negative due to K^+ ion leakage to the luminal side of gastric gland. Due to the energetics of the process, tubulovesicles can associate together but cannot be incorporated into the cell membrane of either the apical or basolateral side *(Fig. 6A)*.

Upon activation, a Cl^- route opens in tubulovesicles. The leakage of Cl^- anions into tubulovesicles decreases positive transvesicle potential and thus activated K^+–H^+–ATP-ase. Internal pH drops below 1 and the lipids become positively charged *(Fig. 6B)*. We are suggesting that at the same time, the membrane potential at the apical face becomes positive. Although the reason for the potential change upon activation is unknown, these changes might result from an electrogenic flow of H^+ or Ca^{2+} ions across the apical
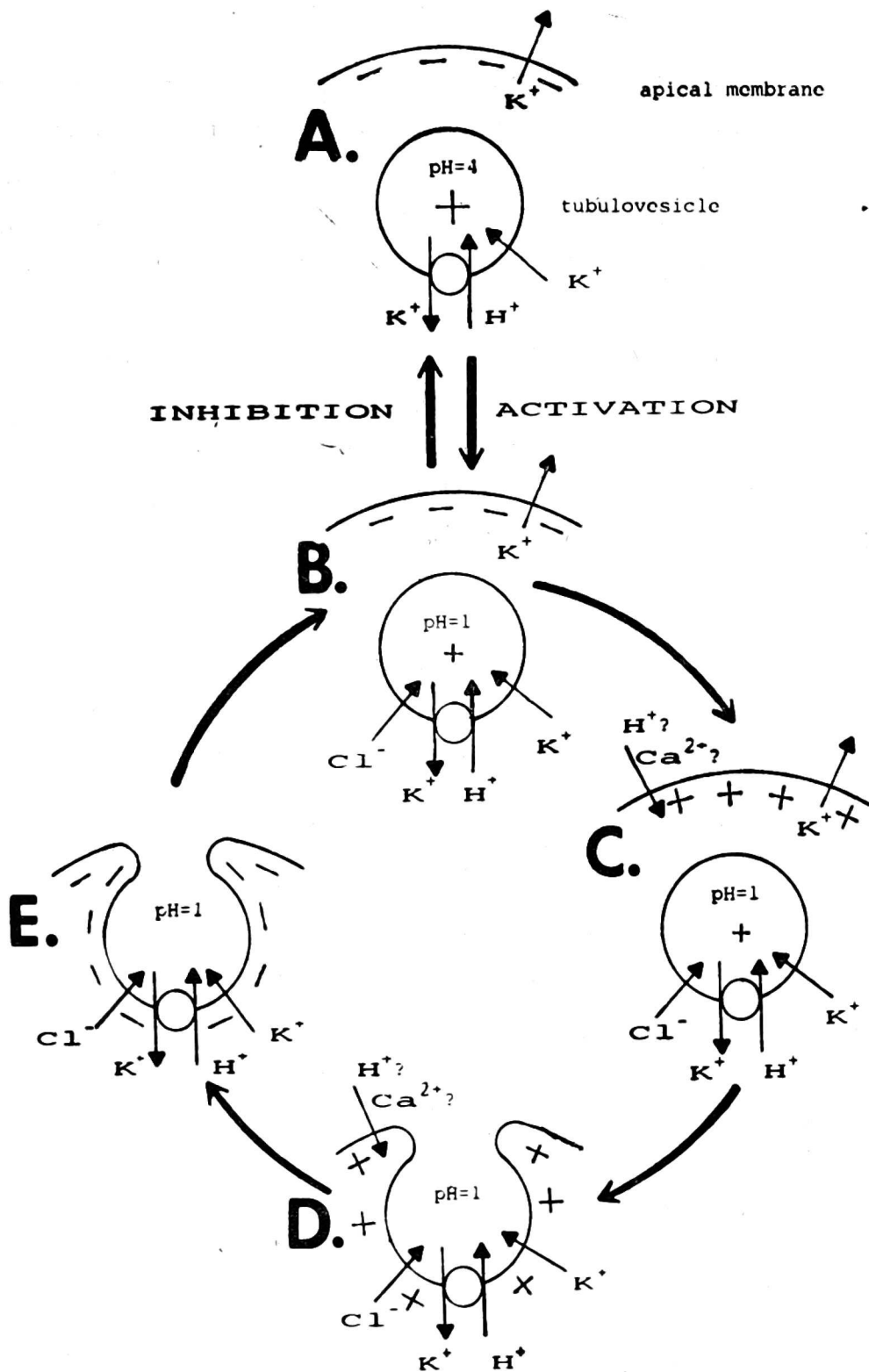
Fig. 6. The membrane recycling in parietal cell. *A: Resting state.* The apical membrane potential is negative. The potential across the tubulovesicle membrane is positive inhibiting K^+-H^+-ATP-ase. pH = 4 inside the tubulovesicle and on luminal face of the apical membrane and, therefore, lipid molecules are net negatively charged. Tubulovesicles can associate together but their incorporation into the basolateral or luminal face of parietal cell is energetically unfavorable. *B: Activation.* Cl^- ions leak into the tubulovesicle causing a decrease in positive vesicle potential that activates K^+-H^+-ATP-ase. pH inside tubulovesicle drops to 1. Lipid molecules become net positively charged. *C: Apical membrane depolarization.* The membrane potential at the apical membrane becomes positive due to an unknown mechanism (e. g. Ca^{2+} or H^+ leakage into the cell). *D: Secretion of HCl.* Due to the positive charge of membrane lipids the incorporation of the tubulovesicles into the apical membrane (but not basolateral one) becomes energetically favorable. *E: Apical membrane HCl prodction.* Proton pump at the apical membrane is active. Membrane potential becomes negative due to K^+ leakage. The change of membrane potential makes energetically favorable the formation of tubulovesicles from the apical membrane. After formation of the tubulovesicles membrane recycling is finished and the system returns to the activated state. Inhibition of acid production results from the inhibition of Cl^- leak across the tubulovesicle membrane.

membrane into the cytoplasm (the other possibility is Cl⁻ export from cytoplasm) *Fig. 6C*. Under experimental conditions, a membrane potential change can be obtained by the use of CCCP treatment of parietal cells in acidic medium or cell polarization by electric current.

Energetic considerations predict that activated tubulovesicles can be incorporated into the apical membrane, but not into basolateral ones since the basolateral membrane potential is negative due to hyperpolarization produced by the secratogogue activation of K^+ channels and stimulation of Na^+–K^+–ATP-ase activity (20, 21). Therefore, the contents of tubulovesicles are released into the luminal side of the gastric mucosa. The surface area of the apical face greatly increases, and number tubulovesicles decreases *(Fig. 6D)*.

The proton pump which was present in tubulovesicles is operating after incorporation at the apical membrane. The membrane potential becomes negative due to K^+ leakage to the luminal face of the apical membrane *Fig. 6E*. Energetic considerations of the process suggest that tubulovesicles are then formed from the apical membrane, and the cycle returns to activated tubulovesicles *(Fig. 6B)*. The resting state of parietal cell *Fig. 6A* is achieved by inhibition of Cl⁻ leakage into the tubulovesicles.

There are numerous experiments with intact gastric tissue in which the secretion of HCl was induced by electric polarization. It has been shown that applying a negative potential to the luminal and a positive potential to the serosal face of gastric tissue causes HCl secretion. The opposite polarization inhibits the secretion (15—19). The polarization which induces secretion also causes the membrane potential across the apical membrane to become positive (inside more positive then outside) and that potential across the basolateral membrane is negative (inside more negative then the outside). In our experiments membrane of the parietal cell might cause the incorporation of the tubulovesicles and release of their contents into the luminal face of the cell. Thus, the effects of the electric field on acid secretion would be similar to those obtained by the use of protonophore in acidic medium.

The working hypothesis that acid secretion by parietal cell depends on changes in the apical membrane potential is highly speculative. Especially it might be presumed that effects of CCCP on aminopyrine accumulation ratios resulted from uncoupling of oxidative phosphorylation by mitochondria. However, since the decreases in aminopyrine accumulation caused by stimultanous addition of CCCP and AP to cell suspensions compared to those in which CCCP was added to incubated cells after achieving the stready-state of AP accumulation were quantitatively similar, it seems unlikely that these changes depended on the decreased cellular ATP essential for acid production. Our suggestion would be strengthened if it could be demonstrated that changes in the morphology of parietal cell secretory system are caused by CCCP

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treatment in acidic media. Therefore, further studies are required, especially electron microscopic quantitive assessment of tubulovesicles after treatment of parietal cells with protonophore.

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